ISSN 0378-4347

VOL. 163 NO. 2 JUNE 11, 1979
(Biomedical Applications, Vol. 5, No. 2)

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

PUBLICATION SCHEDULE FOR 1979

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1978	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	166/1 166/2 167	168/1 168/2	169 170/1	170/2	171 172	173/1 173/2	174/1	174/2 175/1 175/2	The publication schedule for the volumes 176–180 and for further *Chromatographic Reviews* issues (Vol. 165) will be published later.				
Chromatographic Reviews				165/1			165/2						
Biomedical Applications		162/1	162/2	162/3	162/4	163/1	163/2	163/3	163/4	164/1	164/2	164/3	164/4

Scope. The *Journal of Chromatography* publishes papers on all aspects of chromatography, electrophoresis and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications. The section *Biomedical Applications*, which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis (including the publication of normal values); screening and profiling procedures with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; combinations of chromatographic and electrophoretic methods with other physicochemical techniques such as mass spectrometry. In *Chromatographic Reviews*, reviews on all aspects of chromatography, electrophoresis and related methods are published.

Submission of Papers. Papers in English, French and German may be submitted, if possible in three copies. Manuscripts should be submitted to:
The Editor of *Journal of Chromatography*, P.O. Box 681, 1000 AR Amsterdam, The Netherlands
or to:
The Editor of *Journal of Chromatography, Biomedical Applications*, P.O. Box 681, 1000 AR Amsterdam, The Netherlands.
Reviews are invited or proposed by letter to the Editors and will appear in *Chromatographic Reviews* or *Biomedical Applications*. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation.

Subscription Orders. Subscription orders should be sent to: Elsevier Scientific Publishing Company, P.O. Box 211, 1000 AE Amsterdam, The Netherlands. The *Journal of Chromatography, Biomedical Applications* can be subscribed to separately.

Publication. The *Journal of Chromatography* (including *Biomedical Applications* and *Chromatographic Reviews*) has 19 volumes in 1979. The subscription price for 1979 (Vols. 162–180) is Dfl. 2356.00 plus Dfl. 285.00 (postage) (total *ca.* US$ 1288.00). The subscription price for the *Biomedical Applications* section only (Vols. 162–164) is Dfl. 384.00 plus Dfl. 45.00 (postage) (total *ca.* US$ 209.00). Journals are automatically sent by air mail to the U.S.A. and Canada at no extra costs, and to Japan, Australia and New Zealand with a small additional postal charge. Back volumes of the *Journal of Chromatography* (Vols. 1 through 161) are available at Dfl. 140.00 (plus postage). Claims for issues not received should be made within three months of publication of the issue. If not, they cannot be honoured free of charge. For customers in the U.S.A. and Canada wishing additional bibliographic information on this and other Elsevier journals, please contact Elsevier/North-Holland Inc., Journal Information Centre, 52 Vanderbilt Avenue, New York, N.Y. 10017. Tel: (212) 867-9040.

For further information, see page 3 of cover.

Printed in The Netherlands

VOL. 163, NO. 2 JOURNAL OF CHROMATOGRAPHY JUNE 11, 1979

(Biomedical Applications, Vol. 5, No. 2)

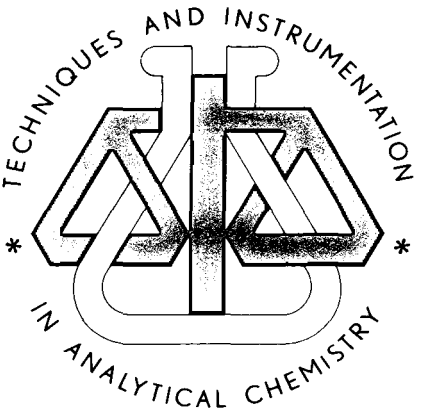
CONTENTS

(Continued overleaf)

Contents (continued)

Notes

Evaluation and Optimization of Laboratory Methods and Analytical Procedures



A Survey of Statistical and Mathematical Techniques

D.L. MASSART, A. DIJKSTRA *and* L. KAUFMAN.

with contributions by S. Wold, B. Vandeginste *and* Y. Michotte

Techniques and Instrumentation in Analytical Chemistry - Volume 1

This book provides detailed treatment, in a single volume, of formal methods for optimization in analytical chemistry. It is a comprehensive and practical handbook which no analytical laboratory will want to be without.

All aspects of optimization are discussed, from the simple evaluation of procedures to the organization of laboratories or the selection of optimal complex analytical programmes. Quantitative discrete analysis as well as qualitative and continuous measurement techniques are evaluated.

The book consists of 30 chapters divided into 5 main parts.The main sections are: Evaluation of the Performance of Analytical Procedures, Experimental Optimization, Combinatorial Problems, Requirements for Analytical Procedures, and Systems Approach in Analytical Chemistry.

This work will be of practical value not only to those involved with optimization problems in analytical chemistry, but also to those in related fields such as clinical chemistry or specialized fields such as chromatography. Because it discusses the application of many mathematical techniques in analytical chemistry, this book will also serve as a general introduction to the new field of Chemometrics.

Oct. 1978 xvi + 596 pages US $57.75/Dfl. 130.00 ISBN 0-444-41743-5

ELSEVIER

P.O. Box 211,
1000 AE Amsterdam
The Netherlands

52 Vanderbilt Ave
New York, N.Y. 10017

The Dutch guilder price is definitive. US $ prices are subject to exchange rate fluctuations.

7113

Journal of Chromatography, 163 (1979) 125—133
Biomedical Applications

CHROMBIO. 323

CAPILLARY COLUMN GAS CHROMATOGRAPHIC PROFILE ANALYSIS OF VOLATILE COMPOUNDS IN SERA OF NORMAL AND VIRUS-INFECTED PATIENTS

ALBERT ZLATKIS, KWAN YOUNG LEE, COLIN F. POOLE and GUNTHER HOLZER

University of Houston, Chemistry Department, Houston, Texas 77004 (U.S.A.)

(Received November 6th, 1978)

SUMMARY

Using a transevaporator sampling technique, the volatile profiles from 70 μl of serum were obtained by capillary-column gas chromatography. The complex chromatograms were interpreted by a combination of manual and computer techniques and a two-peak ratio method devised for the classification of normal and virus-infected sera. Using the K-nearest neighbor approach 85.7% of the unknown samples were classified correctly. Some preliminary results indicate the possible use of the method for the assessment of virus susceptibility.

INTRODUCTION

Recent years have seen a great deal of growth in the development of profile analysis techniques for the recognition of disease disorders [1—20]. A complete profile of all the constituents of a biological fluid is at present impossible with available analytical techniques. The goal at which most workers aim is the development of a complete profile of a selected group of substances (such as steroids, amino acids, etc.) or of compounds with similar physical properties (for example, volatiles). A comparison is then made between the profile obtained with "normal samples" and "pathological samples" to establish any quantitative differences that might be of value for diagnostic purposes. A knowledge of the chemical constitution of abnormal peaks or peaks of abnormal concentration may suggest a possible biochemical reason for the disease and lead to new methods of treatment.

Except in a few cases in which a strong clue exists to implicate the role of causative agents to a disease disorder, most work still relies on a serendipitous finding in a screening program involving the study of many diseases. To maximize the chance of discovery as many substances as possible are included in the profile. The very nature of this approach leads to problems of an analytical nature. For the case of the analysis of volatiles, the current sampling

techniques generate very complex chromatograms which only reveal a full picture of their complexity when high-resolving capillary columns are used for the gas chromatographic separation. The amount of information subsequently generated can no longer be handled by empirical means and the use of pattern recognition techniques and computer sorting are obligatory. Although the selected sample can be characterized as "volatile", under that heading is masked the chemical complexity of the mixture which covers the complete spectrum of polarity. The physical property shared by all components is that they fit into a distinct boiling-point range. The successful use of such techniques as pattern recognition makes high demands on the reproducibility of the profile; a feature which it is all too easy to demonstrate is more often impaired by poor sampling techniques than chromatographic error [21]. The volatiles are present at trace levels in biological fluids, which consist principally of water, so that the sampling technique has to serve as a concentration device capable of reproducibly stripping a diverse range of compounds from a water matrix. For the analysis of urine [1—3, 6, 8—12, 14] sample size and availability are rarely a problem but for serum [7, 16, 20] this is not so, and only relatively small quantities can be obtained from patients.

In an effort to establish the methodology for the early diagnosis of viral diseases, a series of capillary-column chromatographic profiles were obtained using a "transevaporator" sampling technique [22, 23]. The transevaporator is capable of providing reproducible chromatographic profiles of volatile constituents from as little as 10—200 μl of biological fluids and is ideally suited to the analysis of serum samples [23]. The complex chromatographic profiles were analyzed by computer techniques to develop a means of differentiation between normal and virus-infected sera using a two-peak ratio method. A training set was developed and the K-nearest neighbor technique [24—27] used to establish the predictability of virus-infected serum identification. Gas chromatography—mass spectrometry was used to identify the most prominent serum components important to this study.

EXPERIMENTAL

Serum samples

A total of thirty-six serum samples were used from twelve male volunteers who had been exposed to either "England" or "Rhinovirus". These samples form part of a study of immune response to respiratory virus infection conducted by Drs. R. Couch, V. Knight and S. Criswell of the Influenza Research Center (Houston, Texas). Serum samples (200—800 μl) were stored in glass vials at $-20°$ prior to analysis.

The serum samples fall into two categories. The first (Nos. 1—6) were obtained from volunteers who developed clinical symptoms of influenza after virus infection. The second group (Nos. 7—12) were obtained from volunteers who did not develop clinical symptoms after virus infection. Each volunteer provided three serum samples corresponding to a baseline sample taken prior to virus infection (Group I), a serum sample taken one day after exposure to the virus (Group II) and a final sample taken 14—21 days after virus infection (Group III). Group I represents normal serum samples, Group II virus-infected

serum samples (irrespective of whether or not clinical symptoms developed after exposure), and Group III virus-infected serum samples from which all volunteers had recovered clinically from the infection.

Adsorbents and reagents

Porasil E (80—100 mesh) and glass beads (80—100 mesh) were obtained from Analabs (North Haven, Conn., U.S.A.). Prior to use they were washed with distilled diethyl ether and conditioned for 12 h at 280° in a stream of dry helium.

2-Chloropropane (Eastman-Kodak, Rochester, N.Y., U.S.A.) was distilled from phosphorus pentoxide and stored at 5° in the dark prior to use.

Sampling procedure

The serum volatiles were collected from 70 μl of serum sample by the "trans-evaporator" technique described previously [23]. The serum sample is deposited on a Porasil E micro-column and the volatiles stripped from the sample by 2-chloropropane and transferred in the vapor phase to a glass-bead collection column. The volatiles were desorbed thermally at 280° and transferred to a stainless-steel precolumn (30 cm × 1 mm I.D. coated with SF-96) cooled in liquid nitrogen with a helium flow-rate of 7 ml min^{-1} for 10 min. Similarly, the sample is transferred to the analytical column, the first 30 cm of which are cooled in liquid nitrogen by briefly heating the precolumn to about 180° with an air heating gun while passing helium through the system at a rate of 1.5 ml min^{-1}.

Gas chromatography

A Hewlett-Packard 5830A gas chromatograph (Hewlett-Packard, Avondale, Pa., U.S.A.) equipped with flame ionization detectors and connected to a Hewlett-Packard 18850 gas chromatography terminal was used. The serum volatiles were separated on a stainless-steel (100 m × 0.25 mm I.D.) capillary column coated with Witconal LA-23 (Witco, Houston, Texas, U.S.A.) by the dynamic coating method [29]. With a helium carrier gas flow-rate of 1.5 ml min^{-1}, the column was held isothermally at 50° for 10 min and then programmed at 1.5° min^{-1} to 160° and maintained at this temperature for 80 min.

Gas chromatography—mass spectrometry (GC—MS) was performed on an LKB 9000 instrument with a single jet separator and a Perkin-Elmer 900 gas chromatograph. Analytical conditions were as above. Mass spectra were recorded at 70 eV with a scan speed of 4 sec for the mass range 15—300 a.m.u. When possible, identification was confirmed by comparison with standard compounds available in the laboratory, otherwise manual interpretation and comparison to library spectra [30, 31] were used.

Data handling and computer interpretation

The operations that constitute the interpretative procedure are summarized in Fig. 1. Visual inspection was used to identify those peaks common to all chromatograms. The peaks were normalized and this formed the data set for calculation of system variations and variation between individuals within each

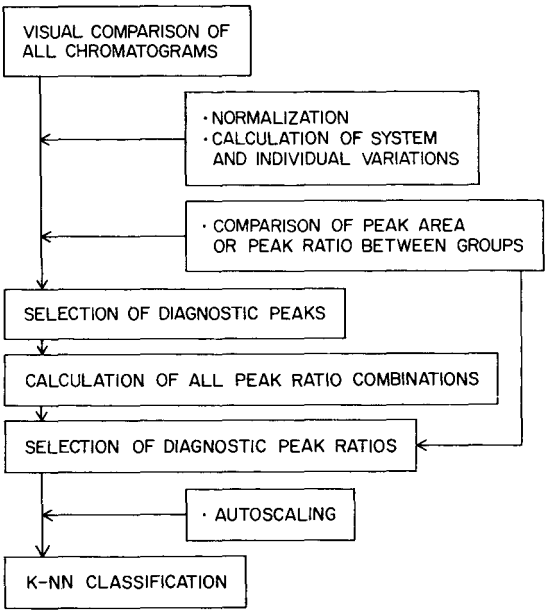


Fig. 1. Schematic diagram of data processing of chromatographic information.

group. A second data set selected on the basis of the magnitude of their difference between groups was used to calculate all possible peak ratio combinations and diagnostic ratios selected. These ratios were autoscaled [28] and used as input for K-nearest neighbor classification. All programs were written in Fortran IV and run on the University of Houston computing center's Honeywell 66/60.

RESULTS AND DISCUSSION

A total of thirty-six virus-infected and normal serum samples as well as six pooled serum samples were studied. The processing of a 70-μl serum sample including sampling by the transevaporator technique, separation by gas chromatography and data input and computation, can be completed within a 2.5-h period. A typical chromatogram obtained from a pooled virus-infected serum sample is shown in Fig. 2A. Peaks identified in the chromatogram by GC—MS are summarized in Table I. The peak numbers in Table I correspond to those marked in Fig. 2. Five of these substances (peaks 5, 8, 10, 13 and 28) have been identified previously in normal serum [20].

The profile of volatile substances in serum is complex, with more than 150 peaks in the chromatogram. To simplify data handling, a visual comparison was made of all chromatograms; peaks due to background and stripping solvent were ignored and 37 peaks which appeared consistently in all chromatograms were selected as the data base. Six replicate analyses of a pooled serum sample were used to establish the magnitude of system variation within the data base (i.e. the effect of experimental variables on reproducibility). All peaks were normalized and the percentage relative standard deviation calculated. Reproducibility depended very much on the compound itself and consequently a

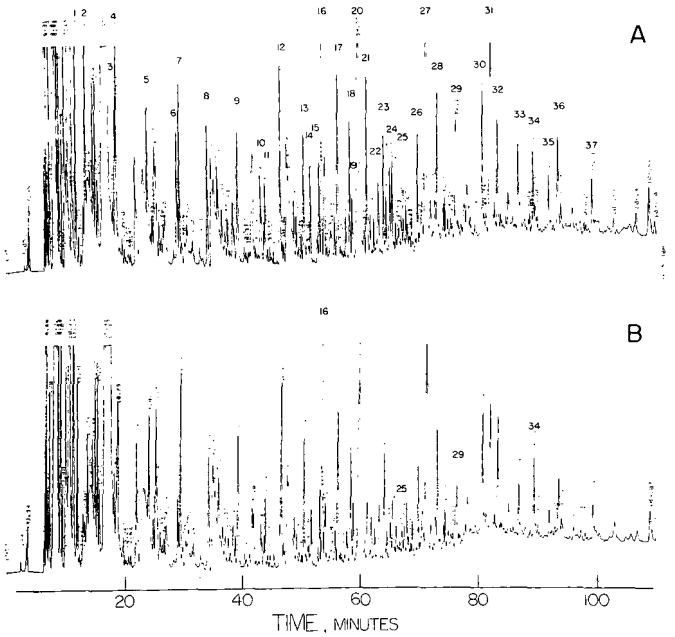


Fig. 2. Profile of organic volatiles by capillary-column gas chromatography. A, virus-infected serum; B, normal serum.

TABLE I

SUBSTANCES IDENTIFIED BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY IN VIRUS-INFECTED SERUM

Components indicated by numbers in Fig. 1.

Peak No.	Compound	Peak No.	Compound
1	2-Methyl-1-hexene	19	2-Octanone
2	Dimethylcyclopentane	20	*n*-Octanal
5	2-Propanol	22	6-Methyl-5-heptene-2-one
7	2-Hexanone	23	5-Nonanone
8	*n*-Hexanal	25	2-Ethylhexanal
10	*n*-Butanol	27	2-Octenol
12	2-Heptanone	28	Benzaldehyde
13	Heptanal	29	2-Ethyl-1-hexanol
14	4-Heptanone	30	2-Decanone
15	4-Octanone	32	*o*-Tolualdehyde
16	6-Methyl-2-heptanone	33	Acetophenone
17	Cyclohexanone and 5-methyl-3-heptanone	34	Trimethyl-2-cyclohexanone

wide variation was found between individual peaks reflecting their different chemical properties. The smallest variation was 6.5% (peak 20), a median value 18.3% (peak 5) and the largest 51.1% (peak 21) relative standard deviation.

Another source of variance is the variability between individuals in any one group. This was calculated for each group in turn for the 37 peaks. All peaks

were normalized and their variance calculated. For example, the 12 serum samples of Group I (normal serum) had a lowest value of 17.1% (peak 1), a median value 32.9% (peak 32) and a highest value of 87.5% (peak 4) relative standard deviation. Results for Group III were similar but Group II showed far less variation.

A second data set was selected, based on the magnitude of the difference between the averaged normalized peak areas for the different groups. the criteria for selection was that the relative standard deviation of the selected peaks must be greater than the variation between individuals in any one group. Seven peaks were found to meet this requirement (peaks 16, 19, 20, 25, 29, 31 and 34).

The normalized peak areas in the second data set can show both negative and positive variation between groups. Under these conditions a ratio of two peaks should prove more sensitive to inter-group differences. Also, if there is an interaction between peaks in each group then peak ratios will be more reliable for classification purposes. The seven normalized peak areas were arranged in ascending order and all possible peak ratios calculated for Groups I and II. The two peak ratios 16/25 and 29/34 were found to be most suitable for sample identification.

The reproducibility of retention time and normalized peak areas for the four selected diagnostic peaks in the pooled serum sample (six replicate analyses) is shown in Table II. Retention times can be reproduced very accurately in the

TABLE II

REPRODUCIBILITY OF NORMALIZED PEAK AREA AND RETENTION TIME WITHIN A POOLED SERUM SAMPLE (6 REPLICATIONS)

Peak No.	Normalized peak area (%)			Retention time (min)		
	Mean	S.D.	C.V. (%)*	Mean	S.D.	C.V. (%)*
16	5.36	1.44	26.9	53.75	0.57	1.0
25	1.34	0.25	18.8	65.82	0.63	0.9
29	4.15	1.37	31.7	76.51	0.62	0.8
34	1.42	0.29	21.0	89.77	0.80	0.9

*Percentage relative standard deviation.

analytical system and this forms an adequate parameter for peak identification. The relative standard deviation of the normalized peak areas between individuals for the four selected peaks is given in Table III for Groups I—III. The variation in peak area for infected serum (Group II) is much less than for normal serum (Group I) and can be more correctly defined. Peak 25 in Group I shows a greater variation than the others due to the inclusion of one extraordinarily large peak in the data set.

To differentiate between normal serum (Group I) and infected serum (Group II) the two peak ratios 16/25 and 29/34 are calculated in Table IV. Visual inspection shows that generally the peak ratio 16/25 decreased upon virus infection (except samples 7, 8 and 9) and that the peak ratio 29/34 increased by virus infection (except samples 2, 3 and 5). However, the use of either peak

TABLE III

VARIATION OF NORMALIZED PEAK AREAS FOR THE SELECTED FOUR PEAKS IN ALL SERUM SAMPLES

Peak No.	Relative standard deviation (%)		
	Before infection (Group 1)	1 day after infection (Group II)	14—21 days after infection (Group III)
16	48.9	32.6	44.2
25	89.9	20.4	47.4
29	36.3	32.7	30.5
34	53.8	30.3	62.9

TABLE IV

SELECTED TWO-PEAK RATIO DATA AT DIFFERENT VIRUS-INFECTED CONDITIONS

Sample No.	Peak ratio (16/25)			Peak ratio (29/34)		
	Before infection	1 day after infection	14—21 days after infection	Before infection	1 day after infection	14—21 days after infection
Serum samples with clinical symptoms after exposure						
1	17.73	4.18	8.73	3.53	7.30	5.54
2	17.93	8.90	9.65	3.85	2.56	3.11
3	24.28	4.77	3.71	44.00	3.24	6.17
4	3.32	2.80	3.67	3.57	4.65	1.88
5	15.78	2.61	5.77	4.15	3.70	3.55
6	17.48	9.51	12.07	0.69	2.43	1.82
Serum samples with no clinical symptoms after exposure						
7	3.38	9.03	8.97	0.99	4.13	1.35
8	6.58	5.05	31.51	0.66	4.40	5.21
9	1.35	1.44	7.56	6.13	7.23	23.22
10	9.24	4.95	2.75	1.94	11.89	1.75
11	33.69	5.59	4.59	1.54	2.52	4.00
12	17.59	8.24	23.15	1.99	8.43	2.22

ratio does not in itself provide a sufficient classification between the two groups. The autoscaled data set of Groups I—III is plotted in two-peak ratio dimensions in Fig. 3. It can be seen that two separate clusters are formed for Groups I and II and thus the two-peak ratios selected are adequate for the identification of normal and infected serum.

In order to test the predictability of the proposed method the K-nearest neighbor approach was used. A randomly chosen training set of ten serum samples (5 normal and 5 infected) was used to assess the predictive accuracy of the two-peak ratio technique using the remaining 14 samples as unknowns. Predictive ability was calculated as the percentage of the 14 unknowns correctly classified. The three nearest neighbor (3-NN) computation assigned 85.7% of the samples correctly (1-NN, 71.4%; 5-NN, 78.5%). Clearly the proposed

method has excellent selectivity for the differentiation of normal and virus-infected serum.

After 14—21 days (Group III) complete recovery from infection is observed clinically. However, the two-peak ratio classification places approximately half the serum samples in the virus-infected category. The implications of this finding can only be speculated on at the moment, especially as normal samples are correctly classified by this technique.

The general usefulness of this profiling technique would be enhanced if it could be used to predict susceptibility to virus infection. The normalized two-peak ratio data for Group I (normal serum) samples are shown in Fig. 4.

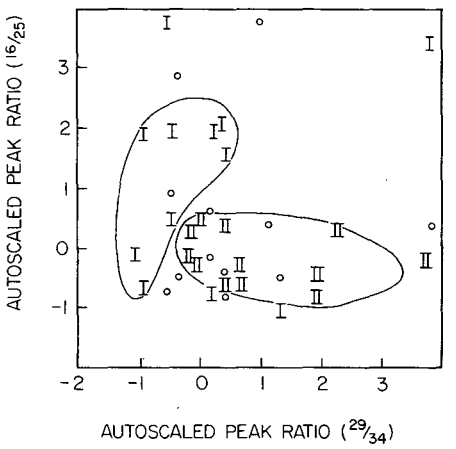


Fig. 3. Autoscaled two-peak ratio diagram. I, normal serum before infection; II, infected sera (24 h); ○, recovered sera (14—21 days after infection).

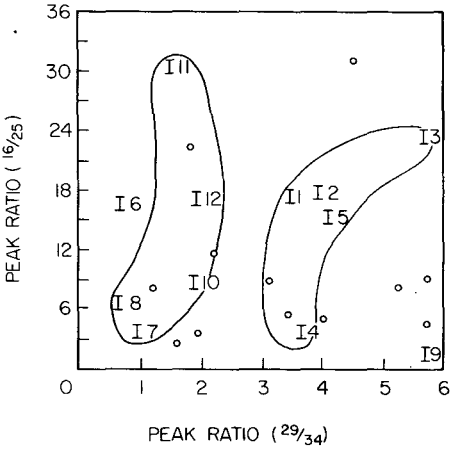


Fig. 4. Two-peak ratio diagram. Samples I1—I6 are normal samples from volunteers who contracted virus infection after exposure; samples I7—I12 the same, except no clinical symptoms developed after exposure.

Samples I1—I6 were normal serum samples from volunteers who upon exposure to virus infection developed clinical symptoms of the disease, and samples I7—I12 did not develop clinical symptoms upon infection. Again two well-defined clusters (with two exceptions: I6, and I9) are formed and demonstrate the possible use of the method for the diagnosis of virus susceptibility. However, the twelve samples available are too small a data base to provide a training set and sufficient unknowns to test the predictability of the method. A much larger sampling program will be required to assess the accuracy of this method for the determination of susceptibility to virus infection.

CONCLUSIONS

The transevaporator sampling technique is shown to be useful for the volatile profile analysis of 70 μl of serum sample. A two-peak ratio method has been developed for the characterization of normal and virus-infected sera with a

percentage predictability of 85.7% of correctly classified unknowns. A similar two-peak ratio method is indicated as a possible means of assessing virus susceptibility.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the support of the National Aeronautics and Space Administration, Life Sciences Directorate, Johnson Space Center, Houston, Texas (Contract NAS9-14629).

REFERENCES

1 A. Zlatkis, W. Bertsch, H.A. Lichtenstein, A. Tishbee, F. Shunbo, H.M. Liebich, A.M. Coscia and N. Fleischer, Anal. Chem., 45 (1973) 763.
2 A. Zlatkis, H.A. Lichtenstein and A. Tishbee, Chromatographia, 6 (1973) 67.
3 H.M. Liebich, O. Al-Babbili, A. Zlatkis and K. Kim, Clin. Chem., 21 (1975) 1294.
4 P.A.G. Malya, J.R. Wright and W.R. Nes, J. Chromatogr. Sci., 9 (1971) 700.
5 B. Krotoszynski, G. Gabriel, H. O'Neill and M.P.A. Claudio, J. Chromatogr. Sci., 15 (1977) 239.
6 R. Teranishi, T.R. Mon, A.B. Robinson, P. Gray and L. Pauling, Anal. Chem., 44 (1972) 18.
7 E. Stoner, D. Cowburn and L.C. Craig, Anal. Chem., 47 (1975) 344.
8 A.B. Robinson and L. Pauling, Clin. Chem., 20 (1974) 962.
9 H. Dirren, A.B. Robinson and L. Pauling, Clin. Chem., 21 (1975) 1970.
10 A.B. Robinson, H. Dirren, A. Sheets, J. Miguel and P.R. Lundgren, Exp. Gerontol., 11 (1976) 11.
11 M. Anbar, R.L. Dyer and M.E. Scolnick, Clin. Chem., 22 (1976) 1503.
12 R.A. Chalmers, M.J.R. Healy, A.M. Lawson, J.T. Hart and R.W.E. Watts, Clin. Chem., 22 (1976) 1292.
13 R.D. Malcolm and R. Leonards, Clin. Chem., 22 (1976) 623.
14 T. Kitagawa, B.A. Smith and E.S. Brown, Clin. Chem., 21 (1975) 735.
15 L. Eldjarn, E. Jellum and O. Stokke, Clin. Chem., 21 (1975) 63.
16 F.W. Bultitude and S.J. Newham, Clin. Chem., 21 (1975) 1329.
17 S.I. Goodman, P. Helland, O. Stokke, A. Flatmark and E. Jellum, J. Chromatogr., 142 (1977) 497.
18 E. Jellum, J. Chromatogr., 143 (1977) 427.
19 H.M. Liebich and O. Al-Babbili, J. Chromatogr., 112 (1975) 539.
20 H.M. Liebich and J. Wöll, J. Chromatogr., 142 (1977) 505.
21 M. Novotny, M.L. McConnell and M.L. Lee, J. Agr. Food Chem., 22 (1974) 765.
22 A. Zlatkis and K. Kim, J. Chromatogr., 126 (1976) 475.
23 K.Y. Lee, D. Nurok and A. Zlatkis, J. Chromatogr., 158 (1978) 377.
24 T.M. Cover and P.E. Hart, IEEE Trans. Inform. Theory, IT-13 (1967) 21.
25 D.W. Peterson, IEEE Trans. Inform. Theory, IT-14 (1970) 26.
26 B.R. Kowalski and C.F. Bender, Anal. Chem., 44 (1972) 1405.
27 J.J. Leary, J.B. Justice, S. Tsuge, S.R. Lowry and T.L. Isenhour, J. Chromatogr. Sci., 11 (1973) 201.
28 H.A. Clark and P.C. Jurs, Anal. Chem., 47 (1975) 374.
29 W. Bertsch, F. Shunbo, R.C. Chang and A. Zlatkis, Chromatographia, 7 (1974) 128.
30 E. Stenhagen, S. Abrahamsson and F.W. McLafferty (Editors), Registry of Mass Spectral Data, Wiley, New York, 1974.
31 Eight Peak Index of Mass Spectra, Mass Spectrometry Data Center, Aldermaston, 2nd ed., 1974.

Journal of Chromatography, 163 (1979) 135—142
Biomedical Applications

CHROMBIO. 332

URINARY PHENYLETHYLAMINE EXCRETION: GAS CHROMATOGRAPHIC ASSAY WITH ELECTRON-CAPTURE DETECTION OF THE PENTAFLUOROBENZOYL DERIVATIVE

KARL BLAU and IRENE M. CLAXTON

Department of Chemical Pathology, Bernhard Baron Memorial Research Laboratories and Institute of Obstetrics and Gynaecology, Queen Charlotte's Maternity Hospital, London W6 0XG (Great Britain)

GÜL ISMAHAN

University of Paris, Unité de Neuropharmacologie, Laboratoire de Physiologie Générale, F-91405 Orsay (France)

and

M. SANDLER

Department of Chemical Pathology, Bernhard Baron Memorial Research Laboratories and Institute of Obstetrics and Gynaecology, Queen Charlotte's Maternity Hospital, London W6 0XG (Great Britain)

(First received November 20th, 1978; revised manuscript received February 23rd, 1979)

SUMMARY

Phenylethylamine was extracted into *n*-hexane from alkaline urine saturated with sodium chloride, and back-extracted into dilute acid. The acid extract was freeze-dried and the residue converted to a pentafluorobenzoyl derivative for analysis by gas chromatography on a column of OV-225 with electron-capture detection. Quantification was achieved by adding an internal standard of tolylethylamine to each sample prior to extraction. Output values in normal subjects and in some patients with phenylketonuria and hyperphenylalaninaemia were in agreement with those in some other recent reports.

INTRODUCTION

Jepson et al. [1] were the first to measure the urinary excretion of phenylethylamine in normal and phenylketonuric subjects, and since that time interest in the endogenous role and metabolism of this amine has gradually increased [2—4]. Disagreement over the range of normal urinary output [1, 5—7] has made it difficult to evaluate claims of changes in pathological condi-

tions. This situation largely reflects differences in methodological approach and points to variations in specificity. We therefore felt it necessary to try to develop a reliable and sensitive gas chromatographic procedure of high specificity, and we describe below such a method based on electron-capture detection of a pentafluorobenzoyl derivative [8].

MATERIALS AND METHODS

Reagents

Solvents were redistilled before use. Phenylethylamine hydrochloride was obtained from Koch-Light Labs. (Colnbrook, Great Britain). *p*-Methylphenylethylamine (tolylethylamine, Aldrich, Gillingham, Great Britain) was converted to its hydrochloride with 1 *M* HCl, evaporated to dryness and recrystallized twice from ethanol. Pentafluorobenzoyl chloride was purchased from Pierce and Warriner (Chester, Great Britain) and stored at −25°.

Preparation of standards

Stock standard solutions of phenylethylamine and tolylethylamine hydrochlorides (1 mg free amine per ml) were made up in 0.01 *M* HCl and stored frozen. Working standards were diluted to a final free amine concentration of 1 μg/ml.

Extraction of amines from urine

Urine (1 ml) in a 15 × 110 mm test-tube, to which tolylethylamine (10 ng) had been added, was mixed on a vortex mixer and adjusted to pH 12.5 with 2.5 *M* NaOH (0.2 ml). After adding solid NaCl (0.3 g), the mixture was extracted with *n*-hexane (2 ml) by vortex mixing for 30 sec and centrifuged at 1500 *g* for 10 min. The upper organic layer was then carefully transferred to a 15-ml conical centrifuge tube with a Pasteur pipette. The hexane layer was back-extracted into 1 *M* HCl (0.4 ml) by vortex mixing for 60 sec, and the mixture was centrifuged at 1500 *g* for 5 min. The lower aqueous layer was transferred to a 2-ml screw-cap glass vial with a Pasteur pipette. Vials in a batch of analyses were put into a rack which was stood on edge in a deep-freeze, so that the extracts were frozen in a nearly horizontal position to increase their surface area. When the extracts were frozen, the whole rack, with the tubes still horizontal, was subjected to freeze-drying with anhydrous "Linde" molecular sieve 3A as desiccant and KOH pellets to absorb acid. This procedure took about 2 h and resulted in barely visible residues. Salt crystals in the vial indicated that aqueous solution had inadvertently been transferred, and such samples were rejected, because alkali in the aqueous layer liberates the free amines, including the internal standard, which are then lost during the drying stage. The amines were isolated in 75—85% yield.

Calibration

Duplicate standards containing increasing ratios of phenylethylamine:tolylethylamine were prepared by measuring, say, 10, 20, 30, 40, 50 μl working standard solution into 2-ml screw-cap vials with 50-μl Hamilton syringes and adding 10 μl tolylethylamine to each. To each vial were also added 10 μl 1 *M*

HCl to prevent amine loss. Solvent was removed rapidly in vacuo and the residues derivatized as described below.

Derivative formation

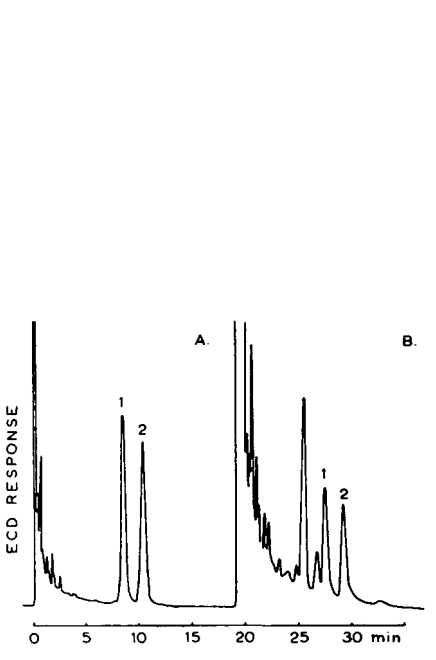
To the residue in each vial, test or standard, was added freshly prepared 1% pentafluorobenzoyl (PFB) chloride in dry diethyl ether (40 μl). The vial was covered with a glass microscope cover-slip and left at 37° for 5 min. Excess reagent was evaporated off in vacuo for about 15 min, after which the procedure was stopped to avoid losses. All-glass derivatization vessels were used because it was found that screw-caps lined with polytetrafluoroethylene caused losses of up to 75% and often gave rise to a large interfering peak of pentafluorobenzoic acid which emerged just before that of the phenylethylamine derivative (Fig. 1). The dried residue of derivatized amine in each vial was dissolved in ethyl acetate (40 μl) for analysis. Where it was desirable to use a reference standard to check the volume injected, the pesticide *p,p*-DDD (1,1-dichloro-2, 2-bis(*p*-chlorophenyl)ethane, Field Instruments, Twickenham, Great Britain) was added to the ethyl acetate used to dissolve the samples, at a concentration of 2.5 μg/l, but this is optional. The use of an injection standard does, however, identify samples with low yields and analyses where the correct volume was not injected for some reason.

Gas chromatography

Analyses were done on a Hewlett-Packard 5713A instrument with an 18713A ^{63}Ni electron-capture detector and a 5709A pulse-modulated electron-capture control module. A coiled pyrex column (180 cm × 0.3 cm I.D.) packed with 3% silicone OV-225 on 80—100 mesh Chromosorb W HP (Applied Science Labs.) was used isothermally at 190°. Injection port temperature was 250° and detector 300°. The carrier gas was 5% methane in argon at 55 ml/min. Before columns were put into service they were conditioned with several injections of the strongest working standard solution.

RESULTS

The isolation procedure was chosen to discriminate against phenolic amines by extracting phenylethylamine at pH 12.5. Various solvents (diethyl ether, ethyl acetate, benzene, chloroform, methylene chloride and hexane) were investigated but hexane proved to be the most suitable because of clean separation, specific extraction and optimal amine recovery. The PFB derivative [8] was chosen as one of the most sensitive for the determination of primary and secondary amines by electron-capture detection [9—11]. PFB chloride reacted rapidly and completely with both phenylethylamine and internal standard, and the resulting derivatives gave clean sharp peaks, well-resolved from other peaks in the urinary profile and from one another. A typical profile from a normal urine extract is shown in Fig. 2. The profile includes unknowns and peaks identified as the PFB derivatives of other amines; our analytical scheme can, in fact, be used to quantify a range of these compounds both endogenous and exogenous and these findings have been reported elsewhere [18, 19]. Table I shows relative retention times of certain amine PFB derivatives and of some reference compounds.



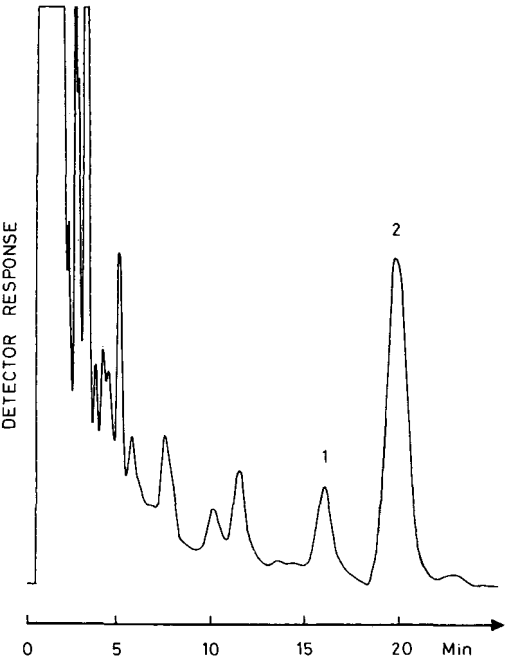


Fig. 1. The effect of polytetrafluoroethylene on derivatization. PFB derivatives of 10 ng each of amine and internal standard were prepared as described in the text. A, Vial was sealed with a glass cover-slip. B, Vial sealed with a PTFE-lined screw-cap. Note reduced peak heights, interfering peaks and slower fall of solvent and reagent peaks in B. Gas chromatography was performed at 210°.

Fig. 2. Gas chromatographic profile of a typical derivatized extract of normal human urine. Peak 1, PFB-phenylethylamine; peak 2, PFB-tolylethylamine. Conditions as in text.

TABLE I

RETENTION TIMES OF PFB-AMINES AND REFERENCE COMPOUNDS, RELATIVE TO PFB-PHENYLETHYLAMINE

Chromatography was carried out on a 180 × 0.3 cm column of 3% OV-225 at 190°. For further details see text.

Compound	Relative retention time
PFB-Phenylethylamine*	1.0
PFB-N-Methylphenylethylamine	0.74
PFB-N-Methylamphetamine	0.75
PFB-Benzylamine	0.79
PFB-Amphetamine	0.82
PFB-*p*-Methylphenylethylamine (tolylethylamine)	1.20
1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT)	1.62
PFB-Phenylpropylamine	1.65
1,1-Dichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p*-DDD)	1.68
PFB-*trans*-Phenylcyclopropylamine (tranylcypromine)	1.87

*Retention time under these conditions was 15.6 min.

The structure of the PFB derivative of phenylethylamine was confirmed mass spectrometrically (Fig. 3). Several urinary extracts were investigated by combined gas chromatography—mass spectrometry under conditions similar to those used for the standard procedure, and gave mass spectra of the PFB-phenylethylamine peak indistinguishable from that in Fig. 3. No evidence of any interfering compound with a retention time equal to that of the phenylethylamine derivative was found in any of these extracts, so that the amine could be determined with high specificity. Fig. 4 shows the linearity of peak height ratios of the phenylethylamine derivative to that of the internal standard, tolylethylamine, plotted against phenylethylamine concentration, using a set of standards as described in the section on calibration.

The internal standard was introduced before analysis, and carried through the entire procedure. It was therefore subjected to the same manipulations as endogenous phenylethylamine, behaved like phenylethylamine during isolation and derivatization, but was resolved from it by gas chromatography. Peak height ratios were calculated and used to determine phenylethylamine concentrations

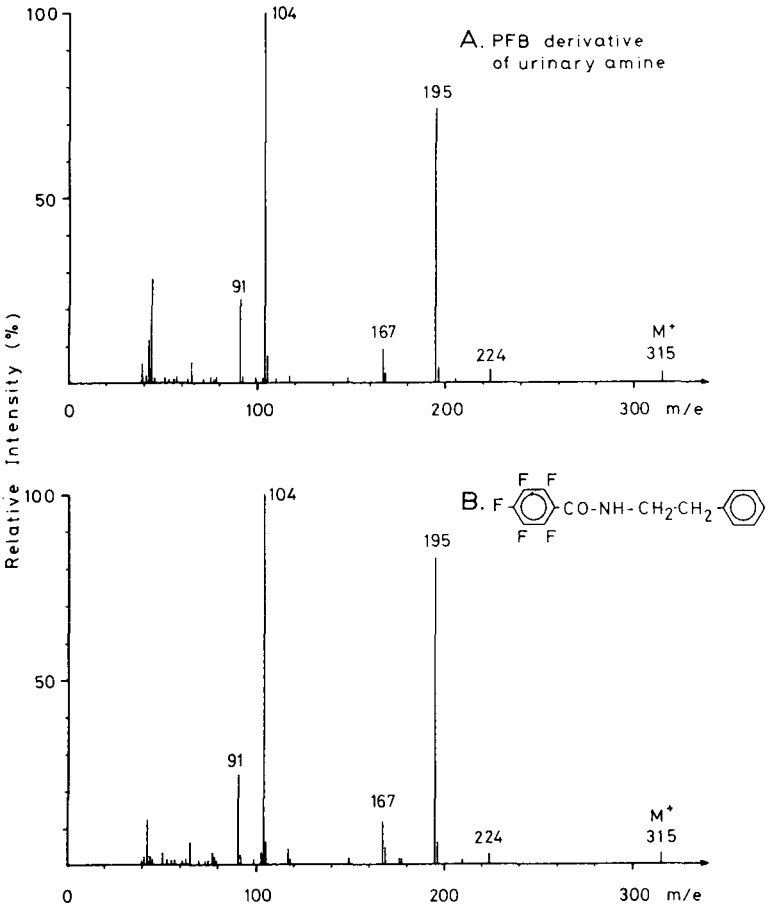


Fig. 3. Electron-impact mass spectra of a PFB derivative of the amine extracted from urine (A) and of authentic PFB-phenylethylamine (B).

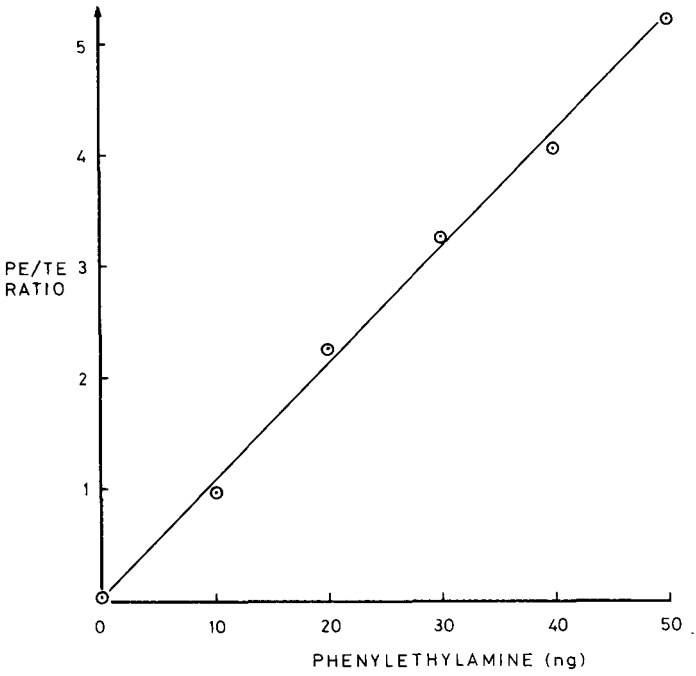


Fig. 4. Typical calibration curve obtained as described in the text. Each point is the average of duplicate determinations. PE, phenylethylamine; TE, tolylethylamine.

in urine samples by reference to the calibration curve (Fig. 4). To check the simplicity and reproducibility of the method, phenylethylamine was determined in ten aliquots of the same urine by a worker who had not previously performed gas chromatographic analyses. The result was 2.31 ± 0.238 μg/l, a standard deviation of just over 10%, and better results have generally been obtained by experienced workers in our department.

Urinary excretion of free phenylethylamine was determined in normal subjects, in a group of adults with hyperphenylalaninaemia not on phenylalanine-depleted diets, and in three infants with confirmed classical phenylketonuria on phenylalanine-reduced diets whose blood phenylalanine concentrations were not well controlled (Table II). These values broadly agree with those given in recent investigations [12—15] in which specific gas chromatographic or mass fragmentographic methods were employed.

DISCUSSION

The present method was developed in response to the need for a sensitive and reliable way of determining phenylethylamine specifically. The analytical conditions were designed accordingly, because earlier reports of high phenylethylamine excretion (for example, refs. 5 and 7) suggested that contributions from interfering substances may have been included. Our procedure proved to be rapid and simple, and has routinely delivered reliable and reproducible results over the last two years. Because of its high sensitivity, only a

TABLE II

URINARY PHENYLETHYLAMINE EXCRETION IN NORMAL SUBJECTS, AND HYPERPHENYLALANINAEMIC AND PHENYLKETONURIC PATIENTS

Patient or subject		Sex	Urinary phenylethylamine		
			μg/l	μg/g creatinine	μg/24 h
Normal subjects					
S.C.		F	1.1	3.7	
C.R.		M	3.8	5.7	
R.J.	1	M	3.0	4.6	
	2		2.3	2.7	
W.W.		M	3.3	1.9	
L.N.		F	2.4	1.9	
C.B.		M	3.9	4.4	
S.B.		F	5.6	2.3	
K.B.	1	M	3.8	8.6	6.5
	2		5.5	6.9	9.0
	3		3.3	4.9	6.2
	4		4.2	4.8	7.2
Hyperphenylalaninaemic patients					
B.J.	1	F	14.0	19.7	21.5
	2		7.6	10.6	12.0
L.A.	1	M	15.2	8.1	15.6
	2		20.0	11.2	20.8
E.A.	1	M	17.0	18.1	33.2
	2		14.2	13.0	30.1
E.B.	1	F	14.8	5.0	
	2		10.0	8.5	
A.F.	1	F	7.0	11.7	
	2		9.6	7.7	
L.M.		M	6.2	5.5	
L.C.		F	8.2	5.6	
Phenylketonuric patients					
V.M.		F	29.9	21.2	
R.S.		M	40.2	33.5	
K.F.		M	51.6	32.2	

small volume of urine is needed, which simplifies experimental manipulations. The method is also applicable to biological fluids other than urine, and with minor modifications has successfully been applied to the determination of phenylethylamine in blood, cerebrospinal fluid and amniotic fluid, and in liver perfusates and tissue homogenates.

The results in Table II are in accordance with previous findings [13, 15] on the general relationship between urinary phenylethylamine output and blood phenylalanine concentration. The phenylketonuric children had the highest phenylethylamine output, while that of the hyperphenylalaninaemic subjects, like their phenylalanine blood levels, was intermediate between the values of the normal subjects and the higher values of phenylketonuric patients.

Comparison of the urinary phenylethylamine excretion relative to creatinine with 24-h phenylethylamine output when both values were available (Table II) indicates that the correlation between them is not very good. This is due partly

to diurnal fluctuation in blood phenylalanine concentrations affecting random urinary phenylethylamine values [16] and partly also to variations in the pH at which the urine was excreted by the kidneys [17]. For reliable metabolic studies one should ideally collect 24-h urine samples and also arrange for metabolic acidification of the urine by ammonium chloride ingestion.

The agreement of the more recent reports with the present results makes it clear that specific chromatographic methods must be used to resolve phenylethylamine from interfering substances in order to achieve reliable quantitative results. The availability of such methods should now enable us to obtain more dependable indications of the significance of phenylethylamine both in normal physiology and in pathological conditions.

ACKNOWLEDGEMENTS

We thank Dr. G.S. King for performing the mass spectrometry and Dr. G.P. Reynolds for valuable discussions.

REFERENCES

1 J.B. Jepson, W. Lovenberg, P. Zaltzman, J.A. Oates, A. Sjoerdsma and S. Udenfriend, Biochem. J., 74 (1960) 5P.
2 M. Sandler, S.B. Carter, B.L. Goodwin and C.R.J. Ruthven, in E. Usdin and M. Sandler (Editors), Trace Amines and the Brain, Marcel Dekker, New York, 1976, p. 233.
3 H.C. Sabelli, R.L. Borison, B.I. Diamond, H.S. Havdala and N. Narasimhachari, Biochem. Pharmacol., 27 (1978) 1707.
4 M. Sandler and G.P. Reynolds, Lancet, i (1976) 70.
5 E. Fischer, H. Spatz, J.M. Saavedra, H. Reggiani, A.H. Miro and B. Heller, Biol. Psychiat., 5 (1972) 139.
6 A.A. Boulton, and L. Milward, J. Chromatogr., 57 (1971) 287.
7 A.D. Mosnaim, E.E. Inwang, J.H. Sugerman and H.C. Sabelli, Clin. Chim. Acta, 46 (1973) 407.
8 G.R. Wilkinson, Anal. Lett., 3 (1970) 289.
9 E. Änggård and A. Hankey, Acta Chem. Scand., 23 (1969) 3110.
10 S.B. Matin and M. Rowland, J. Pharm. Sci., 61 (1972) 1235.
11 A.C. Moffat, E.C. Horning, S.B. Matin and M. Rowland, J. Chromatogr., 66 (1972) 255.
12 J.W. Schweitzer, A.H. Friedhoff and R. Schwartz, Biol. Psychiat., 10 (1975) 277.
13 G.P. Reynolds and D.O. Gray, Clin. Chim. Acta, 70 (1976) 213.
14 H. Andersen and C. Braestrup, Scand. J. Clin. Lab. Invest., 37 (1977) 33.
15 G.P. Reynolds, J.W.T. Seakins and D.O. Gray, Clin. Chim. Acta, 83 (1978) 33.
16 R.D. Feigin, W.R. Beisel and R.W. Wannemacher, Amer. J. Clin. Nutr., 24 (1971) 329.
17 G.P. Reynolds, P.M. Ceasar, C.R.J. Ruthven and M. Sandler, Clin. Chim. Acta, 84 (1978) 225.
18 G.P. Reynolds, J.D. Elsworth, K. Blau, M. Sandler, A.J. Lees and G.M. Stern, Brit. J. Clin. Pharmacol., 6 (1978) 542.
19 M.B.H. Youdin, J.K. Aronson, K. Blau, A.R. Green and D.G. Grahame-Smith, Psychol. Med., in press.

Journal of Chromatography, 163 (1979) 143—150
Biomedical Applications

CHROMBIO. 336

DETERMINATION OF PLASMA AND URINARY CORTISOL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING FLUORESCENCE DERIVATIZATION WITH DANSYL HYDRAZINE

TAKAO KAWASAKI, MASAKO MAEDA and AKIO TSUJI*

School of Pharmaceutical Sciences, Showa University, Hatanodai, Shinagawa-ku, Tokyo (Japan)

(Received November 14th, 1978)

SUMMARY

A method is described for the determination of cortisol in human plasma and urine by high-performance liquid chromatography using fluorophotometric detection. After extraction with methylene chloride, cortisol is labelled with dansyl hydrazine, and then separated by high-performance chromatography. The eluate is monitored by a fluorophotometer at 350 nm (excitation) and 505 nm (emission). The optimum conditions for the determination, such as HCl and dansyl hydrazine concentrations, reaction time and reaction temperature, and for the eluent of high-performance liquid chromatography, are discussed. Linearity of the fluorescence intensity (peak height) with the amount of cortisol was obtained between 0.5 and 60 ng. The recoveries for 50 and 100 ng of added cortisol were 98.7 and 95.4% for plasma, and 96.4 and 90.6% for urine, respectively. Comparison with a radioimmunoassay gave a correlation coefficient of 0.978. The proposed method is suitable for the routine analysis of cortisol in plasma and urine.

INTRODUCTION

Cortisol is an important secretory product of the adrenal cortex, and represents a good parameter for adrenal activity. Many methods have been proposed for the determination of cortisol in biological samples, including thin-layer and gas—liquid chromatography [1], spectrophotometry [2], fluorimetry [3] and radioimmunoassay [4]. In certain instances, these methods may be considered lacking in either sensitivity, specificity, reproducibility or convenience of analysis. It has recently been demonstrated that high-performance liquid chromatography (HPLC) is suitable for the analysis of cortisol and corticosterone in biological materials [5—9]. On the other hand, fluorigenic labelling techniques have been used for several years in conjunction with thin-layer chromatography

*To whom correspondence should be addressed.

[10, 11], and are now being used also to good advantage in HPLC [12, 13]. In this study, dansyl hydrazine was used to derivatize the carbonyl group of cortisol. The fluorescent derivative was then separated and detected by HPLC equipped with a fluorimeter. This method has been applied to the quantitative determination of cortisol in human plasma and urine.

EXPERIMENTAL

Materials

All chemicals used were of reagent grade and obtained from commercial sources.

Apparatus

An Hitachi Model 634 high-speed liquid chromatograph and an Hitachi Model 204 fluorophotometer equipped with xenon lamp and flow cell were used.

Chromatographic conditions

A 250 mm × 4 mm I.D. stainless-steel column fitted with an on-column injection port was used. The column packing was Hitachi gel No. 3042 (silica gel, particle size 5 μm). The column was packed by a slurry method. The eluent system, dichloromethane–ethanol–water (948:35:17) was used according to the description of Hesse et al. [14, 15]. The eluent was prepared as follows: 17 ml of redistilled water and 35 ml of ethanol were made up to 1000 ml with dichloromethane. The mixture was shaken for 1 h at room temperature and then allowed to stand until the emulsion had cleared. After separation, the organic phase was ready for use. The column was equilibrated at 35° with the above eluent; the flow-rate was 1 ml/min, which corresponded to a pressure drop of 25 kg/cm^2 in the column used. The effluent was monitored with a fluorimeter at an excitation wavelength of 350 nm and emission wavelength of 505 nm.

Reagent solutions

Dansyl hydrazine solution: A 0.02% (w/v) solution of dansyl hydrazine was prepared by dissolving 2 mg of dansyl hydrazine (Sigma, St. Louis, Mo., U.S.A.) in 10 ml of ethanol, and stored at 4°.

Hydrochloric acid–ethanol solution: The solution was prepared by mixing 0.9 ml of concentrated HCl and 500 ml of ethanol.

Cortisol stock solution: One mg of cortisol (Merck, Darmstadt, G.F.R.) was dissolved in redistilled water and made up to 100 ml. The resultant stock solution was stored at 4°.

Cortisol working standard solution: One ml of the cortisol stock solution was transferred into a 50-ml or 100-ml volumetric flask and made up to volume with redistilled water before use.

Procedure

Extraction of cortisol from plasma and urine: Half a millilitre of well-mixed plasma or 1.0 ml of urine was transferred to a screw-cap culture tube. Half a

millilitre of water, 0.1 ml of 2 *N* NaOH solution, and 10 ml of methylene chloride were added to the tube, which was then capped tightly. The mixture was stirred vigorously for 1 min by a Vortex-type mixer, and centrifuged for 5 min at 10,000 *g*. The aqueous layer was removed with a Pasteur pipette, and the organic layer was washed with 1 ml of 0.1 *N* H_2SO_4 by stirring for 30 sec, after which the aqueous layer was removed again. After drying by the addition of 2 g of anhydrous Na_2SO_4, exactly 7.0 ml of the organic layer were evaporated to dryness at 40° under a stream of nitrogen gas.

Labelling reaction: The residue in the test-tube was dissolved by adding 0.1 ml of HCl—ethanol and then mixed with 0.01 ml of dansyl hydrazine solution. After standing for 30 min, the solvent was evaporated to dryness under a stream of nitrogen gas. The labelled residue was dissolved in 50 μl of chloroform; 20 μl of this were injected into the chromatograph.

RESULTS

Fluorescence spectrum

Fig. 1 shows the excitation and emission spectra of the dansyl hydrazone of cortisol in chloroform which was prepared by preparative thin-layer chromatography after reaction. The excitation wavelength maximum was 350 nm and the emission maximum 505 nm. The fluorescence intensity of dansyl derivatives was affected by the polarity of the solvent. The solvent effects on the fluorescence were therefore examined before the analytical technique was

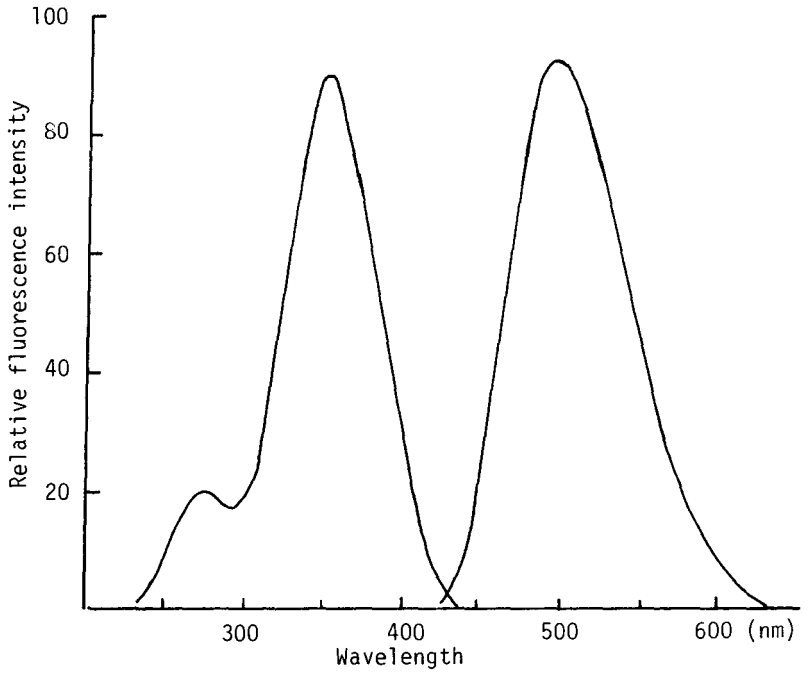


Fig. 1. Excitation and emission spectra of the fluorescent dansyl hydrazone of cortisol in chloroform. Excitation maximum, 350 nm; emission maximum, 505 nm.

developed. Among the solvent systems tested, methylene chloride gave the most intense fluorescence, and the polar solvents such as methanol, ethanol, etc., gave a lower fluorescence intensity. Although the eluate used in this paper contained a small amount of ethanol and water, the fluorescence intensity of the dansyl hydrazone of cortisol in it was almost the same as that in methylene chloride.

Concentration of HCl and dansyl hydrazine

Dansyl hydrazine reacted with cortisol to form hydrazone in acidic medium. As shown in Fig. 2, the reaction time to reach a maximum and constant peak

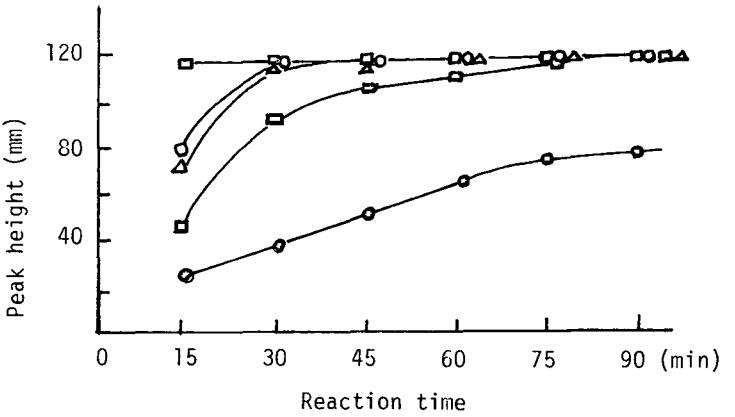


Fig. 2. Effect of HCl concentration and reaction time on peak height (fluroescence intensity). The amounts of concentrated HCl in 500 ml of ethanol were: 3.5 ml (□), 1.8 ml (○), 0.9 ml (△), 0.45 ml (■), and 0.225 ml (●). Injected volume: 5 μl of the final solution containing 35 ng of cortisol.

height decreased with increased HCl concentration in ethanol. Using 3.6 ml of concentrated HCl in 500 ml of ethanol, the peak height reached a constant value within 15 min, but unkown subpeaks appeared in the chromatogram. Therefore, the HCl—ethanol solution containing 0.9 ml of concentrated HCl in 500 ml of ethanol was used for the reaction to prevent the formation of by-products with the acid. The peak height reached a maximum at 30 min with this HCl—ethanol mixture so that the reaction time was held for 30 min. Fig. 3 shows the relationship between the fluorescence intensity (as peak height) and the ratio of dansyl hydrazine to cortisol. The peak height increased with increasing ratio, up to 3:1, and then became constant. In practice, the constant peak height of cortisol extracted from 0.5 ml of normal plasma was obtained by using more than 1 μg of dansyl hydrazine, whereas the peak of cortisol could not be separated from that of the excess dansyl hydrazine when 5 μg were used. Therefore, 0.01 ml of dansyl hydrazine solution containing 2 mg per 10 ml of ethanol was used for labelling.

Reaction temperature

The reaction temperature was set at room temperature, because the reaction rate was independent of temperature between 0° and 60°.

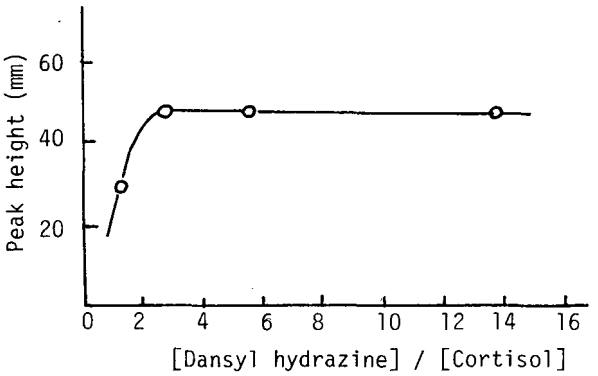


Fig. 3. Effect of dansyl hydrazine concentration on peak height (fluorescence intensity). Injected volume: 15 μl of the final solution containing 15 ng of cortisol.

Selection of eluent

Many solvent systems were examined in order to select the suitable eluent. An organic layer of methylene chloride—ethanol—water (948:35:17) was found to be suitable when used with an Hitachi gel No. 3042 column. The organic layer saturated completely with water by shaking at least for 1 h should be used as the eluent because the peak of cortisol splits into two peaks when unsaturated solvent is used. The chromatogram presented in Fig. 4 shows a good separation of cortisol and the other related steroids by this eluent.

Working curve and selectivity

Linearity of the fluorescence intensity (peak height) with the amount of cortisol injected was obtained between 0.5 and 60 ng. The detection limit of cortisol was about 0.2 ng from this working curve. Then, using 0.5 ml of plasma and 1.0 ml of urine as samples, the detection limits are 100 ng and 50 ng per 100 ml, respectively.

Recovery and reproducibility

The extraction procedure was carried out with methylene chloride as

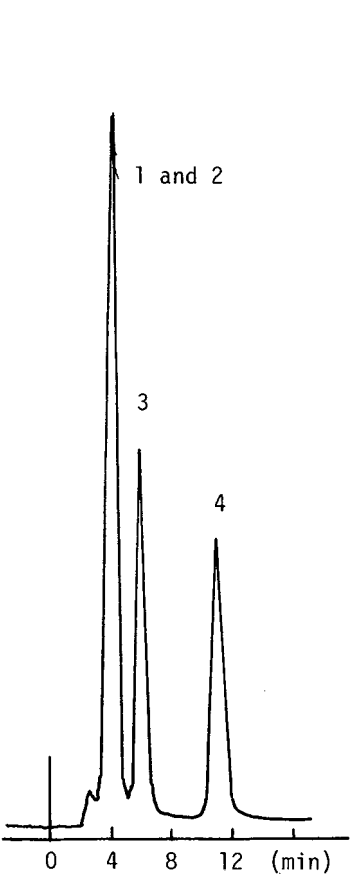
TABLE I

RECOVERY OF CORTISOL ADDED TO PLASMA AND URINE PRIOR TO EXTRACTION

Sample	Added (ng)	Found (ng)	Recovery (%)	*n*	C.V. (%)
Plasma	0	1.8*	—	5	15.7
(0.5 ml)	50	51.1	98.7	8	3.3
	100	97.1	95.4	12	3.7
Urine	0	49.6*	—	10	4.9
(1.0 ml)	50	97.8	96.4	10	4.4
	100	140.2	90.6	10	6.2

*Value before spiking.

solvent. The cortisol-free plasma was presented by treatment of dexamethazone. In recovery tests the method was applied to samples to which had been added 50 ng and 100 ng of cortisol per 0.5 ml of cortisol-free plasma and 1.0 ml of normal urine. The reproducibility was determined by carrying out 8—12 analyses, with the results shown in Table I. Some typical chromatograms are shown in Fig. 5. The large peak appearing in front of cortisol is due to excess dansyl hydrazine and the fluorescent compounds in the sample, but no peaks interfering with cortisol were observed. The same chromatograms were also obtained with the urine samples.



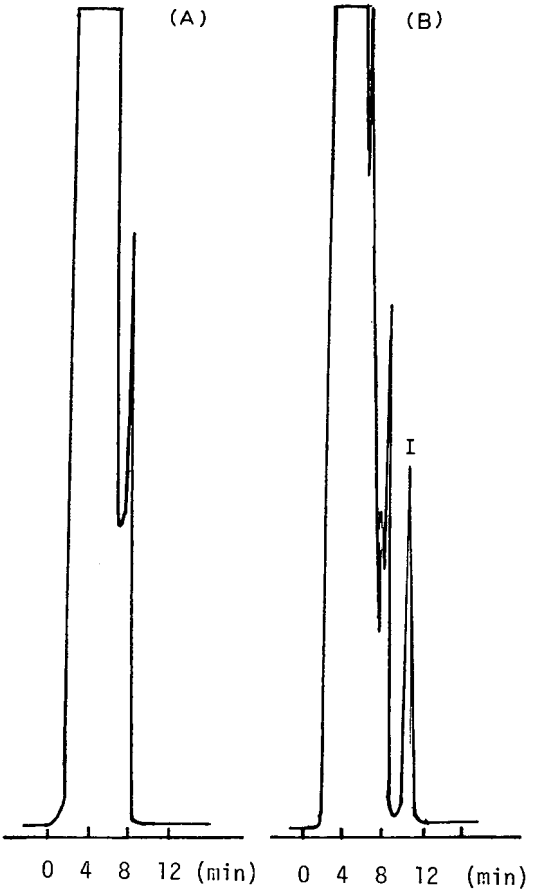


Fig. 4. Chromatogram of dansyl hydrazone derivatives of Δ^4-3-oxo-steroids. 1, Corticosterone; 2, 11-desoxy-17-hydroxycorticosterone; 3, cortisone; 4, cortisol. Column (250 mm × 4 mm I.D.) prepared with Hitachi gel No. 3042; flow-rate, 1 ml/min; solvent system, dichloromethane—ethanol—water (948:35:17); wavelengths, 350 nm (excitation) and 505 nm (emission).

Fig. 5. Typical chromatograms of plasma samples. (A) Cortisol-free plasma presented by treatment of dexamethazone. (B) Normal human plasma. Peak I: cortisol. Assay procedure and chromatographic conditions are given in the text.

Comparison with radioimmunoassay

The reliability of this method for the determination of cortisol in plasma or urine was assessed by comparing the results with those obtained by radioimmunoassay. As illustrated in Fig. 6, the values obtained by each method were in excellent agreement, the coefficient of correlation for cortisol samples in the range 5—250 ng/ml of plasma being 0.978. The values obtained by this method were slightly higher than those obtained by radioimmunoassay.

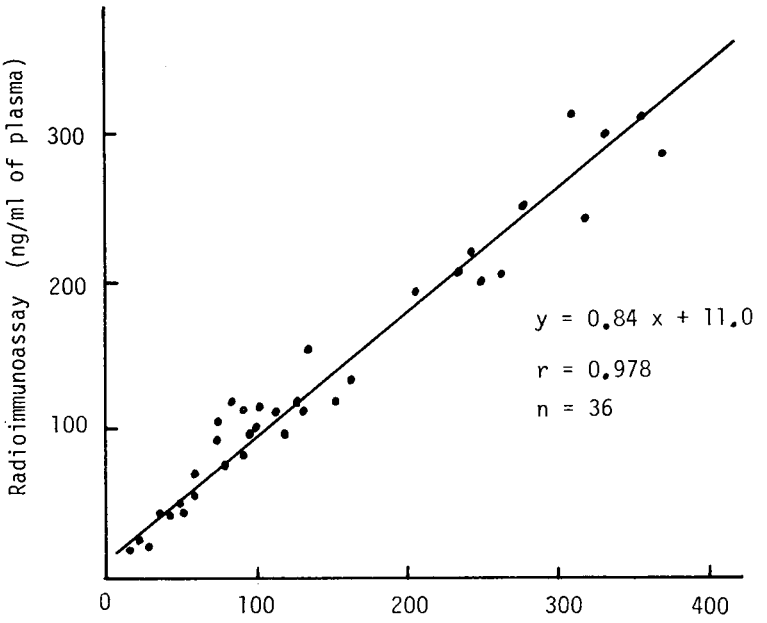


Fig. 6. Comparison of results obtained with fluorescence HPLC and radioimmunoassay.

DISCUSSION

Several reports on the use of HPLC to assay cortisol in biological fluids [5—9] have been published, but the sensitivities of most of them were too low due to the use of an UV detector. Fluorophotometric detection is more sensitive than the UV detection method. Fluorophotometry of cortisol could not be used in conjunction with HPLC because concentrated acids were used as reaction media. Recently, a fluorophotometric determination of Δ^4-3-oxo-steroids using aluminium salt and isonicotinylhydrazine has been developed by Horikawa et al. [16]. They stated that the reaction would be utilized in the detection of Δ^4-3-oxo-steroids in the eluate after separation by HPLC.

The advantages of fluorescence derivatization in liquid chromatography of trace amounts of substances in biological fluids are evident. Frei and Lawrence [17] used dansyl hydrazine as a fluorescent labelling reagent for carbonyl compounds. Chayen et al. [18] developed a thin-layer chromatographic determination of oxo-steroids, such as testosterone and cortisol, by using dansyl hydrazine. However, both methods have not been applied to biological samples.

In this paper, we have developed a clinically useful method for the determination of plasma or urine cortisol by HPLC with a pre-labelling technique using dansyl hydrazine. The optimum conversion to the fluorescent dansyl hydrazone was obtained by carrying out the reaction with 0.02% dansyl hydrazine—ethanol solution and HCl—ethanol (containing 0.9 ml of concentrated HCl per 500 ml of ethanol). The chromatographic conditions were chosen to give acceptable resolution between dansyl hydrazone of cortisol and the fluorescent co-extractives from plasma or urine samples in the shortest possible analysis time.

The detection limit of this method is 0.2 ng or better, depending on the efficiency of the fluorescence detector and the final injection volume. The sensitivity of the method is superior to those of other HPLC methods using an UV detector. Trefz et al. [6] reported that the cortisol values in plasma obtained by HPLC were slightly higher than those obtained from radioimmunoassay. Although the slightly higher values than those of radioimmunoassay were also obtained using our method, there was no interference in the clinical application being continued in our laboratory. The results will be reported in the near future.

ACKNOWLEDGEMENT

This work was supported by a grant from the Ministry of Education (Japan) which is gratefully acknowledged.

REFERENCES

1 E. Heftmann, Chromatography of Steroids, Elsevier, Amsterdam, Oxford, New York, 1976, p. 101.
2 S. Görög and Gy. Szász, Analysis of Steroid Hormone Drugs, Elsevier, Amsterdam, Oxford, New York, 1978, p. 330.
3 S. Udenfriend (Editor), Fluorescence Assay in Biology and Medicine, Academic Press, New York, Vol. I, 1962, p. 349 and Vol. II, 1969, p. 455.
4 G.E. Abraham and W.D. Odell, in F. Peron and B. Caldwell (Editors), Immunological Methods in Steroid Determination, Appleton-Century-Crofts, New York, 1970, p. 87.
5 W. Wortmann, C. Schnabel and J.C. Touchstone, J. Chromatogr., 84 (1973) 396.
6 F.K. Trefz, D.J. Byrd and W. Kochen, J. Chromatogr., 107 (1975) 181.
7 G. Schwedt, H.H. Bussemas and Ch. Lippmann, J. Chromatogr., 143 (1977) 259.
8 J.H.M. van den Berg, Ch.R. Mol, R.S. Deelder and J.H.H. Thijssen, Clin. Chim. Acta, 78 (1977) 165.
9 D. Ishii, K. Hibi, K. Asai, M. Nagaya, K. Mochizuki and Y. Mochida, J. Chromatogr., 156 (1978) 173.
10 L.P. Penzes and G.W. Oertel, J. Chromatogr., 51 (1970) 325.
11 N. Seiler, J. Chromatogr., 63 (1971) 97.
12 J.F. Lawrence and R.W. Frei, Chemical Derivatization in Liquid Chromatography, Elsevier, Amsterdam, Oxford, New York, 1976, p. 153.
13 Z. Tamura, N. Ishibashi, Y, Okura, T. Tanimura and A. Tsuji (Editors), LC-Fluorescence Analysis, Kodanshya Scientific, Tokyo, 1978, p. 63 (in Japanese).
14 C. Hesse and W. Hovermann, Chromatographia, 6 (1973) 345.
15 C. Hesse, K. Pietrizik and D. Hotzel, Z. Klin. Chem. Klin. Biochem., 12 (1974) 193.
16 R. Horikawa, T. Tanimura and Z. Tamura, Anal. Biochem., 85 (1978) 105.
17 R.W. Frei and J.F. Lawrence, J. Chromatogr., 83 (1973) 321.
18 R. Chayen, D. Davir, S. Gould and A. Harell, Anal. Biochem., 42 (1971) 283.

Journal of Chromatography, 163 (1979) 151—160
Biomedical Applications

CHROMBIO. 329

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FREE AND TOTAL POLYAMINES IN HUMAN SERUM AS FLUORESCAMINE DERIVATIVES

MASAAKI KAI

Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

TOMOFUMI OGATA

The Third Department of Internal Medicine, Faculty of Medicine, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

and

KOICHI HARAGUCHI and YOSUKE OHKURA*

Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

(Received December 28th, 1978)

SUMMARY

A highly sensitive and simple fluorimetric method for the determination of free and total polyamines, spermidine, spermine, putrescine and cadaverine, in human serum by high-performance liquid chromatography is described. The polyamines, obtained after clean-up of deproteinized serum by Cellex P column chromatography, are converted to their fluorescamine derivatives in the presence of nickel ion which inhibits the reaction of interfering amines with fluorescamine, and the derivatives are separated simultaneously by reversed-phase chromatography (LiChrosorb RP-18) with a linear gradient elution. The lower limits of detection are 10 and 5 pmole for spermine and the others in 0.5 ml of serum, respectively.

INTRODUCTION

In recent years, it has been described that the amounts of polyamines, spermidine (Spd), spermine (Spm) and putrescine (Put), are increased in growing tissues [1—3] and cancerous tissues [4—8] and thus the amounts of the

*To whom correspondence should be addressed.

amines are useful for the evaluation of the efficacy of chemotherapy on cancer [9—11]. Therefore, the quantitation of the polyamines in physiological fluid has become of interest in the possible early diagnosis of malignancy. Several methods have been reported (e.g., enzymatic, ion-exchange chromatographic, gas chromatographic, thin-layer chromatographic and liquid chromatographic methods), which were recently reviewed by Seiler [12]. Among the methods, ion-exchange chromatographic methods using amino acid analysers are popular, but have a limited sensitivity.

The concentrations of the polyamines, Spd, Spm, Put and cadaverine (Cad) in serum are normally very low, and so totals of free and acetylated polyamines have usually been measured after treatment of deproteinized serum with acid-hydrolysis [6, 13, 14]. The quantitation of free polyamines was described in a few reports [15—17].

Samejima [18] described a sensitive fluorimetric method coupled with high-performance liquid chromatography (HPLC) of fluorescamine (FA) derivatives of the polyamines. In the method, the derivatives of Put and Cad and those of Spd and Spm were separately subjected to HPLC because of insufficient resolution of the derivative of Spd from that of Cad. Therefore, the method in practical use required a column chromatographic separation of Put and Cad from Spd and Spm prior to reaction with FA [16].

Recently, we found that FA derivatives of the four polyamines and 1,6-hexanediamine can be separated simultaneously by reversed-phase HPLC, and that the reaction of many biogenic amines other than the polyamines with FA is inhibited considerably by nickel ion, which may serve to minimize the interference from those amines. This paper describes a simple HPLC method with fluorescence detection for the determination of free and total polyamines in human serum utilizing the above findings. 1,6-Hexanediamine is used as an internal standard.

EXPERIMENTAL

Materials and reagents

Sera were obtained from Kyushu University Hospital and healthy volunteers in our laboratory. All chemicals were of reagent grade, unless otherwise noted. Double-distilled water and solvents were used. FA and Cellex P were obtained from Japan Roche (Tokyo, Japan) and Bio-Rad (Richmond, Calif., U.S.A.), respectively.

Cellex P column. Cellex P (H^+ form, 0.9 mequiv./g) should be washed to remove contaminant before use according to the Kremzner and Wilson method [19] with some modifications as follows. Wash 20 g of Cellex P successively with ca. 500 ml of 0.2 *M* sodium hydroxide, ca. 400-ml portions of water, 0.5 *M* hydrochloric acid, water, 1 *M* sodium chloride, water, 1 *M* sodium carbonate, water, 0.1 *M* sodium hydroxide, water, ethanol and water, ca. 800-ml portions of 0.5 *M* sodium chloride and water, and finally ca. 400-ml portions of 0.2 *M* and 0.01 *M* sodium phosphate buffers (pH 6.0). Suspend the washed Cellex P in ca. 500 ml of 0.01 *M* sodium phosphate buffer (pH 6.0) and store in a refrigerator. When required for use, add 0.01 *M* sodium phosphate buffer (pH 6.0) to three times the settling volume of Cellex P after

standing for 1 h, mix and pour onto a glass column (15 × 0.5 cm I.D.) to be 3.0 cm height after settling.

Mobile phase in HPLC. For methanol linear gradient elution, two solutions (A and B) are required. Prepare first a salt solution by dissolving 13.37 g of ammonium chloride, 15.76 g of sodium benzenesulfonate and 1 g of acetic acid in ca. 400 ml of water, adjust the pH to 4.0 at 25° with 1 *M* sodium hydroxide and dilute with water to 500 ml (ammonium chloride, 0.5 *M*; sodium benzenesulfonate, 0.175 *M*). Prepare solution A by mixing 100 ml of the salt solution with 175 ml of water and 225 ml of methanol, and solution B by mixing 100 ml of the salt solution with 400 ml of methanol. Both solutions are de-gassed in the usual manner before use.

Apparatus

The HPLC system consisted of a Mitsumi liquid chromatograph equipped with a 013 high-pressure piston pump and a 7120 syringe loading sample injector, a Hitachi solvent gradient device, a Hitachi 203 spectrofluorimeter equipped with a Hitachi flow-cell unit (cell volume, 20 μl) and a Hitachi 056 recorder (chart speed, 2.5 mm/min). The fluorescence intensity was monitored at the emission wavelength of 490 nm with the excitation wavelength set at 390 nm. A stainless-steel column (15 × 0.4 cm I.D.) was packed with Li-Chrosorb RP-18, 5 μm (Japan Merck, Tokyo, Japan) as previously described [20]. The column can be used for more than 350 injections with only a small decrease in the theoretical plate number. The column temperature was kept at 30 ± 0.5° by circulation of water through a glass-jacket fitted with the column to obtain certain retention times of peaks in the chromatogram. A Hitachi-Horiba M-7 pH meter was used.

Procedure

Place 0.5 ml of serum in a centrifuge tube, add 0.1 ml of 4 nmole/ml 1,6-hexanediamine as an internal standard and dilute with water to 1.0 ml. Add 0.1 ml of 3 *M* perchloric acid and centrifuge at 1200 *g* for 5 min. Adjust the pH of the supernatant to 7.0 ± 0.5 with 1.5 *M* potassium hydroxide, cool in ice-water and remove potassium perchlorate thus formed by centrifugation. For the sample solution of free polyamines, add 1.0 ml of 0.1 *M* sodium phosphate buffer (pH 7.0) to the supernatant, mix 0.5 ml of chloroform and 0.3 ml of methanol, and centrifuge at 1200 *g* for 5 min. Use the aqueous layer as the sample solution. For the sample solution of total polyamines, place the supernatant obtained after the removal of potassium perchlorate in a screw-cap culture tube, add 1.0 ml of concentrated hydrochloric acid, heat at 115° in an oil-bath for 12 h and dry in vacuo at room temperature to remove excessive acid. Dissolve the residue in 1.0 ml of water and then treat in the same way as in the preparation of the sample solution for free polyamines.

Load the sample solution on the Cellex P column, wash the column by pouring successively 2.0 ml of 0.01 *M* sodium phosphate buffer (pH 6.0), 1.0 ml of water and 1.5 ml of 0.05 *M* sodium chloride and then elute the polyamines and 1,6-hexanediamine with 2.0 ml of 3 *M* sodium chloride. To the eluate, add 0.5 ml of 0.4 *M* borate buffer (pH 9.0) and 0.2 ml of 20 m*M* nickel(II) sulfate, and add 0.5 ml of 1 m*M* FA solution in anhydrous acetone with vigorous mixing.

To the reaction mixture, add 1.0 ml of 0.3 *M* succinic acid, 1 g of sodium chloride and 0.4 ml of ethyl acetate. Shake the mixture vigorously for ca. 30 sec on a Vortex-type mixer and allow to stand for ca. 1 min. Transfer the organic layer to a test-tube, add 3.0 ml of cyclohexane, and extract the FA derivatives with 0.2 ml of 0.4 *M* borate buffer (pH 10.0) with mixing on a Vortex-type mixer for ca. 30 sec. Prepare reagent blanks by treating 0.5 ml of water instead of serum in the same way as for free and total polyamines. Inject 100 μl of each of the extracts of the tests and blanks into the chromatograph, elute by linear gradient elution between solution A and B during 25 min at a constant flow-rate of 1.0 ml/min, and monitor the fluorescence intensities of the eluates.

Calculate the ratios of net peak heights due to the individual polyamines in serum to that due to the internal standard. Read the concentrations of free or total polyamines from working curves prepared as follows. Treat 0.5 ml each of pooled serum added with 0 and 0.05—0.4 ml of the standard solution containing 0.5 nmole/ml each of the polyamines (0 and 25—200 pmole added, respectively) in the same way as for free and total polyamines in serum and plot the ratios of net peak heights due to each polyamine added to that due to the internal standard against the amounts of each polyamine added.
Typical working curves thus obtained are shown in Fig. 1.

RESULTS AND DISCUSSION

HPLC conditions

FA derivatives of the polyamines and 1,6-hexanediamine can be completely separated in 20 min by reversed-phase HPLC with linear gradient elution with a methanol concentration between 45 and 80% (Fig. 2).

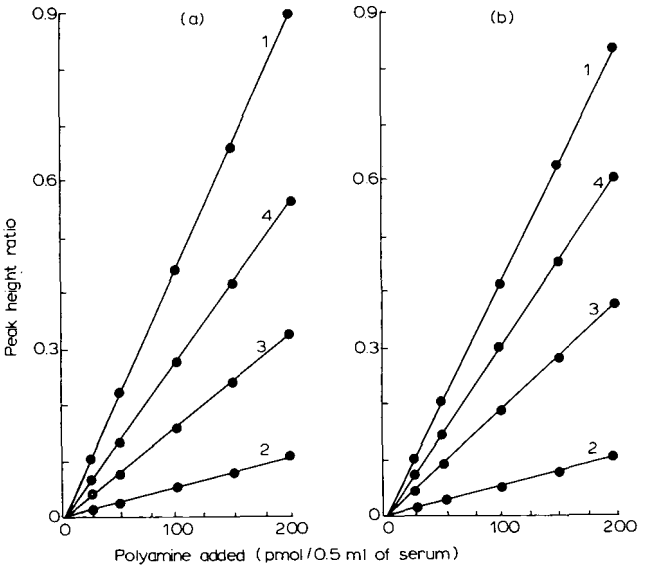


Fig. 1. Working curves for (a) free and (b) total polyamines. Curves: 1, spermidine; 2, spermine; 3, putrescine; 4, cadaverine.

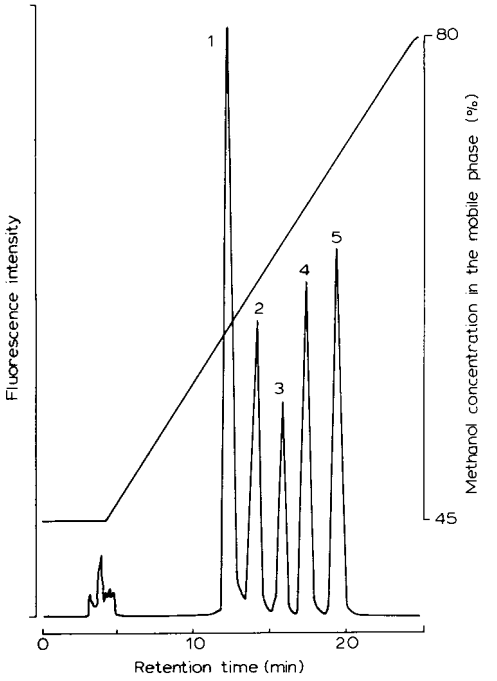


Fig. 2. Chromatogram of fluorescamine derivatives of spermidine, spermine, putrescine, cadaverine and 1,6-hexanediamine. A 0.5-ml aliquot of a standard mixture containing 4 nmole each of the amines was treated as described under *Procedure*. Peaks: 1, spermidine; 2, spermine; 3, putrescine; 4, cadaverine; 5, 1,6-hexanediamine.

The concentrations of ammonium chloride and sodium benzenesulfonate in the mobile phase affect the resolution of the FA derivatives. At the concentration of ammonium chloride lower than the prescribed concentration, 0.1 *M*, the separation of the derivatives of Spd, Spm and Put is incomplete, but the order of their elution remains unchanged, while the higher concentration causes delay of the elution. At the concentration of sodium benzenesulfonate lower or higher than the prescribed concentration, 0.035 *M*, the peaks due to Spd and Spm overlap or those due to Spm and Put nearly overlap, respectively. The pH of the mobile phase affects the elution pattern of the derivatives. The peaks due to Spd and Spm are split into more than two peaks, at pH higher than 8, suggesting that those derivatives exist as more than two fluorescent species in the alkaline medium [16]. At pH higher than 6, the derivatives of Put and Cad elute earlier than that of Spm, and at pH lower than 3, an insufficient resolution of the peaks due to Spd and Spm occurs. At the recommended pH 4, the derivatives of Spd and Spm fluoresce most intensely and the five peaks are well separated.

The relative retention times of FA derivatives of FA-reacting biological amines examined are tabulated in Table I. FA derivatives of many amines eluted earlier than those of the polyamines, but the derivatives of several amines eluted closely to those of the polyamines, which may interfere with the determination of the polyamines if present in the sample solution for

TABLE I

RELATIVE RETENTION TIMES* OF FLUORESCAMINE DERIVATIVES OF THE POLYAMINES AND OTHER BIOLOGICAL AMINES

To a mixture of 2.0 ml of water and 0.5 ml of 0.4 *M* borate buffer (pH 9.0) together with 10 nmole of the amine, 0.5 ml of 1 m*M* fluorescamine solution was added, and 100 µl of the mixture was subjected to HPLC.

Compound	Relative retention time	Compound	Relative retention time
Guanidine	not detectable	Alanine	0.33 0.10**
Agmatine	not detectable	Dopamine	0.35
Histidine	0.05	Tyramine	0.47
Aspartic acid	0.07	Methionine	0.54 0.09**
Glutamic acid	0.07	Tryptophan	0.64
Threonine	0.07	Spermidine	0.71
Tyrosine	0.07	Ornithine	0.76 0.06**
Citrulline	0.07	Spermine	0.77
Glutathione	0.07	1,3-Diaminopropane	0.80 0.59**
Carnosine	0.07	Leucine	0.81
Glycine	0.08	Angiotensin II	0.82 0.54**
Valine	0.08	Lysine	0.82 0.67 0.08**
Histamine	0.08	Isoleucine	0.82 0.35 0.10**
Urea	0.08	Putrescine	0.83
DOPA	0.09	Phenylalanine	0.88 0.37**
Serotonin	0.09	Cadaverine	0.89
Noradrenalin	0.25	1,6-Hexanediamine	1.00

*Retention time of fluorescamine derivative of 1,6-hexanediamine was taken as 1.00.
**Two or three peaks were observed in the chromatograms.

HPLC. Therefore the sample solution should be free from such interfering amines.

Sample solutions for HPLC

The extraction of free and total polyamines, the deproteinization of serum and the acid hydrolysis of acetylated polyamines can be accomplished by the usual methods [6, 13, 14].

Prior to loading of the deproteinized sample solution or the solution obtained after the acid hydrolysis on the Cellex P column for clean-up, lipids in the solutions should be removed to effuse smoothly through the column. This could be done by extraction with chloroform and methanol. The interfering amines held in the column after loading, which may be very large amounts as compared with those of the polyamines, can be removed by successive elution with 0.01 *M* sodium phosphate buffer, water and 0.05 *M* sodium chloride. The polyamines and the internal standard are then eluted with 3 *M* sodium chloride.

Very small amounts of primary amines, which eluted near the FA derivatives of the polyamines in HPLC (see Table I), remained even after the clean-up and occluded as contaminants from the reagents, glass-ware and laboratory environment in the sample solutions and interfered with the determination of

nmole to pmole quantities of the polyamines. Nickel ion inhibits the derivatization of the interfering amines with FA by 40—93%, but not that of the polyamines, under the conditions of the derivatization at pH 9.0 (Table II), which is useful to minimize the interference of those amines in the procedure. No effect of the nickel ion is observed if added after the reaction with FA.

The derivatization of individual polyamines is weakly affected by the pH of the buffer used in the presence of a high concentration of sodium chloride as in the present procedure. Although the most intense fluorescence is obtained in the reaction at pH 8.8—9.5 for Put, Cad and 1,6-hexanediamine, and at pH 7.0—7.5 for Spd and Spm, a small difference between the fluorescence intensities obtained at pH 9.0 and 7.5 is observed. Thus a borate buffer of pH 9.0 was used in the procedure.

FA derivatives of the polyamines and 1,6-hexanediamine in the reaction mixture can be completely extracted with a small amount of ethyl acetate after acidification of the mixture with succinic acid in the presence of sodium chloride. When the extract is subjected to HPLC, an insufficient separation of the derivatives is observed because of broadening of the peaks. A high resolution of the peaks is achieved by back-extraction of the FA derivatives in the extract with a small amount of a borate buffer of pH 10.0. This extraction serves to concentrate the FA derivatives and the peak heights thus observed are ca. 7 times those obtained without the extraction.

TABLE II

INHIBITORY EFFECT OF NICKEL ION ON THE REACTION OF BIOLOGICAL AMINES WITH FLUORESCAMINE

To 10 nmole of the amine in a mixture of 2.0 ml of 3 *M* sodium chloride and 0.5 ml of 0.4 *M* borate buffer (pH 9.0) mixed with 0.2 ml of 20 m*M* nickel sulfate solution or water, 0.5 ml of 1 m*M* fluorescamine solution was added, and the fluorescence intensity was measured with a Hitachi MPF-4 spectrofluorimeter in a cell of 1 × 1 cm at the emission wavelength of 480 nm with the excitation wavelength at 394 nm.

Compound	% Inhibition	Compound	% Inhibition
Histidine	100	Lysine	54
Glutathione	95	Phenylalanine	41
Angiotensin II	93	Isoleucine	40
Histamine	93	Agmatine	0
Tryptophan	91	Spermidine	0
Ornithine	88	Spermine	2
1,3-Diaminopropane	58	Putrescine	0
Leucine	58	Cadaverine	0
Arginine	56	1,6-Hexanediamine	0

Determination of polyamines in serum

Fig. 3 shows typical chromatograms obtained with normal serum and the reagent blank under the conditions of the procedure without acid hydrolysis. The polyamines and 1,6-hexanediamine (internal standard) are completely separated after 11—20 min, in which range of time no peaks ascribable to interfering amines are observed. Three small peaks in the reagent blank (Fig.

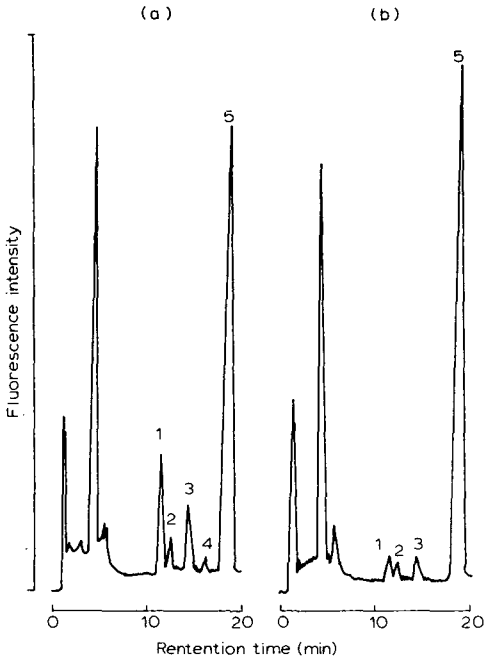


Fig. 3. Chromatograms of fluorescamine derivatives of free polyamines in normal serum (a) and the reagent blank (b), obtained according to the procedure. Peaks: 1, spermidine; 2, spermine; 3, putrescine; 4, cadaverine; 5, 1,6-hexanediamine.

3b, peaks 1—3) have exactly the same retention times as those of Spd, Spm and Put in serum sample (Fig. 3a, peaks 1—3), and increase in their heights when the blank added with Spd, Spm and Put is used, indicating that the peaks are caused by those polyamines occluded as contaminants. When a reagent blank prepared without chromatography on the Cellex P column is subjected to HPLC, only weak peaks ascribable to those amines are observed. This fact suggests that the amines are due mainly from the Cellex P column chromatographic treatment. More intense peaks are observed even if a fresh column packed with commercial Cellex P without washing, as described in the Experimental section, is used, which may interfere with a highly sensitive determination of the polyamines in serum sample. The same is also true when a column used for serum sample or reagent blank is re-used. When the reagent blank is repeatedly applied on the LiChrosorb RP-18 column, constant heights of the peaks due to the polyamines as contaminants are obtained. Thus, in calculation of peak height ratios in the procedure, peak heights of peaks 1—3 in the reagent blank should be subtracted from those in the serum sample.

Linear relationships were observed between the ratios of the peak heights of the polyamines to those of the internal standard and the amounts of the polyamines added in the range of 10—200 pmole to 0.5 ml of serum, both in free and total polyamines (cf. Fig. 1), and no change of the slopes in the graphs of the relationships was observed depending on the serum used. These facts indicate that the present internal standard method permits the deter-

TABLE III

FREE AND TOTAL POLYAMINE CONCENTRATIONS IN NORMAL HUMAN SERUM

Age	Free polyamine* (pmole/ml)				Total polyamine* (pmole/ml)			
	Spd	Spm	Put	Cad	Spd	Spm	Put	Cad
22	150	80	200	0	360	80	260	10
22	90	0	140	10	300	10	260	50
25	80	0	120	0	200	30	160	40
25	10	0	130	20	130	0	290	50
26	60	0	70	10	360	50	300	20
27	10	0	110	10	130	0	190	30
28	50	30	120	10	220	110	270	50
28	180	60	150	0	350	120	400	50
30	10	30	60	10	130	0	180	100
30	80	0	200	0	200	60	350	200
Mean	70	20	130	10	240	50	270	60

*Spd, spermidine; Spm, spermine; Put, putrescine; Cad, cadaverine.

mination of free and total polyamines in serum over wide ranges of their concentrations.

The recoveries of the polyamines added to 0.5 ml of serum in the amounts of 25—200 pmole were 97 ± 3% for free and total Put and Cad, and 75 ± 5% for free and total Spd and Spm. The recoveries were calculated from the determined values obtained with the fortified serum and standard mixtures of the polyamines treated as in the procedure.

The lower limits of detection for Spm and the others are 10 and 5 pmole in 0.5 ml of serum, respectively. The limit was defined as the amount giving a signal-to-noise ratio of 2. The precision of the method was examined by performing 10 assays separately on free and total polyamines in pooled serum containing free Spd, Spm, Put and Cad at 50, 30, 120 and 10 pmole/ml, and total ones at 220, 110, 270 and 50 pmole/ml, respectively. The coefficients of variation were 7, 8, 2 and 3% for free Spd, Spm, Put and Cad, and 4, 6, 2 and 2% for total ones, respectively.

The concentrations of free and total polyamines in the sera of 10 healthy men (22—30 years) determined by this method are shown in Table III. The mean values of individual polyamines are in good agreement with the published data [6, 13—17].

The proposed method is very sensitive and simple to perform, and therefore may be applicable for routine use.

ACKNOWLEDGEMENTS

Kind encouragement from Professor H. Ibayashi of the Faculty of Medicine Kyushu University and a Grant-in-Aid for Scientific Research from the Minis-

try of Education, Science and Culture of Japan are gratefully acknowledged. The skilful assistance of Mrs. Y. Ogata (nee Ino) is also acknowledged.

REFERENCES

1 J. Jänne, A. Raina and M. Siimes, Acta Physiol. Scand., 62 (1964) 352.
2 D.H. Russell, V.J. Medina and S.H. Snyder, J. Biol. Chem., 245 (1970) 6732.
3 D.H. Russell and J.B. Lombardini, Biochim. Biophys. Acta, 240 (1971) 273.
4 D.H. Russell and C.C. Levy, Cancer Res., 31 (1971) 248.
5 H.G. Williams-Ashman, G.L. Coppoc and G. Weber, Cancer Res., 32 (1972) 1924.
6 K. Nishioka and M.M. Romsdahl, Clin. Chim. Acta, 57 (1974) 155.
7 M. Tsuji, T. Nakajima and I. Sano, Clin. Chim. Acta, 59 (1975) 161.
8 N. Seiler, U. Lamberty and M. J. Al-Therib, J. Neurochem., 24 (1975) 797.
9 D.H. Russell and S.D. Russell, Clin. Chem., 21 (1975) 860.
10 B.G.M. Durie, S.E. Salmon and D.H. Russell, Cancer Res., 37 (1977) 214.
11 D.H. Russell, Clin. Chem., 23 (1977) 22.
12 N. Seiler, Clin. Chem., 23 (1977) 1519.
13 L.J. Marton, D.H. Russell and C.C. Levy, Clin. Chem., 19 (1973) 923.
14 L.J. Marton and R.L.Y. Lee, Clin. Chem., 21 (1975) 1721.
15 K.D. Cooper, J.B. Shukla and O.M. Rennert, Clin. Chim. Acta, 73 (1976) 71.
16 K. Samejima, M. Kawase, S. Sakamoto, M. Okada and Y. Endo, Anal. Biochem., 76 (1976) 392.
17 K.D. Cooper, J.B. Shukla and O.M. Rennert, Clin. Chim. Acta, 82 (1978) 1.
18 K. Samejima, J. Chromatogr., 96 (1974) 250.
19 L.T. Kremzner and I.B. Wilson, J. Biol. Chem., 238 (1963) 1714.
20 Y. Tsuruta, K. Kohashi and Y. Ohkura, J. Chromatogr., 146 (1978) 490.

Journal of Chromatography, 163 (1979) 161—167
Biomedical Applications

CHROMBIO. 322

MEASUREMENT OF MEPHENYTOIN (3-METHYL-5-ETHYL-5-PHENYLHYDANTOIN) AND ITS DEMETHYLATED METABOLITE BY SELECTIVE ION MONITORING

WAYNE YONEKAWA* and HARVEY J. KUPFERBERG

Epilepsy Branch, Neurological Disorders Program, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)

(First received October 24th, 1978; revised manuscript received February 5th, 1979)

SUMMARY

Mephenytoin (3-methyl-5-ethyl-5-phenylhydantoin) and its demethylated metabolite Nirvanol (5-ethyl-5-phenylhydantoin) were measured by a selective ion monitoring technique. This method was used in the analysis of both compounds in plasma from a patient receiving 50 mg and 400 mg of mephenytoin in single oral doses. Both compounds were extracted from plasma and ethylated prior to analysis by electron-impact mass spectrometry. Alkylation, using ethyl iodide in 2-butanone, occurred in the N-1 and N-3 positions of the hydantoin ring when concentrated KOH was added to the reaction mixture. The lower limits of quantitation for mephenytoin and Nirvanol were 10 ng/ml and 50 ng/ml, respectively, and were found to be reproducible within 8% upon repeated quantification.

INTRODUCTION

Mephenytoin (3-methyl-5-ethyl-5-phenylhydantoin; Mesantoin) was introduced in 1945 for the treatment of a wide range of seizure types, including generalized tonic—clonic seizures, elementary partial seizures, and complex partial seizures, It has action against pentylenetetrazol convulsions and abolishes the tonic phase of maximal electroshock seizures in man and animals [1].

Butler [2, 3] and Butler and Waddell [4] showed that the primary metabolite of mephenytoin in dogs and man was the demethylated product, 5-ethyl-5-phenylhydantoin, Nirvanol (Fig. 1). The liver was found to be the principal organ of metabolism. Mephenytoin is converted to Nirvanol, which is subsequently converted to the primary urinary metabolite 5-ethyl-5-(*p*-hydroxyphenyl)hydantoin. Kupferberg and Yonekawa [5] found that the anticonvul-

*To whom correspondence should be addressed.

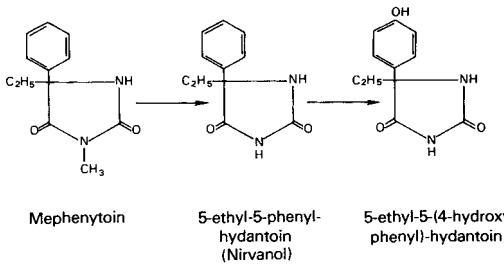


Fig. 1. The metabolic conversion of mephenytoin to Nirvanol and its hydroxylation to 5-ethyl-5-(4-hydroxyphenyl)hydantoin.

sant activity of mephenytoin in mice remained essentially unchanged during a 2-h period following a single intraperitoneal injection of mephenytoin. Mephenytoin levels in the plasma and brain rose quickly and fell at later times, whereas the Nirvanol levels rose slowly and reached their maximum at 2 h. The conclusion was that mephenytoin was responsible for the early activity and Nirvanol for the later activity.

Few methods for the simultaneous analysis of mephenytoin and Nirvanol have been reported. Friel and Troupin [6] have described a gas chromatographic method for the analysis of several anticonvulsant drugs, including mephenytoin and Nirvanol. Derivative formation was accomplished by "flash-heater" ethylation, using triethylphenylammonium hydroxide as the ethyl donor. The ethylated products were nearly resolved on an OV-1 column. The only interfering substance was the ethylated product of mephobarbital, which could not be resolved from Nirvanol. The levels of mephenytoin were found to be near the method's lower limits of detection in patients on chronic mephenytoin therapy. Kupferberg and Yonekawa [5] analyzed mephenytoin and Nirvanol as the trimethylsilyl derivatives. The lower limit of sensitivity was approximately 1 μg for each compound extracted from biological tissue. Baseline resolution was difficult despite the use of a variety of phases of differing polarity.

Quantitative selective ion monitoring (SIM) overcomes many of the above-mentioned problems. Low levels of drugs can be quantitated with an increased degree of specificity. Baseline resolution of closely eluting peaks of compounds differing in concentration by as much as twenty times can be quantitated by monitoring the different *m/e* of each compound.

This paper describes a quantitative SIM assay for mephenytoin and Nirvanol following plasma extraction and derivatization. Selected compounds of analogous structure which undergo a similar alkylation reaction were chosen as internal standards. The method was then applied to the quantitation of mephenytoin and Nirvanol in plasma from a patient receiving 50 mg and 400 mg of mephenytoin in single oral doses.

MATERIALS AND METHODS

Chemicals and reagents

Chloroform, hexane, and methanol "distilled in glass" were obtained from

Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Ethyl iodide was obtained from Eastman Labs. (Rochester, N.Y., U.S.A.). 2-Butanone and 5-methyl-5-phenylhydantoin (MPH) were obtained from Aldrich (Milwaukee, Wisc., U.S.A.). Mephenytoin (MEPH) and Nirvanol (EPH) were supplied by Sandoz Pharmaceuticals (Hanover, N.J., U.S.A.). 3-Methyl-5-cyclopropyl-5-phenylhydantoin (MCPH) was supplied by Abbott Labs. (North Chicago, Ill., U.S.A.).

Extraction of mephenytoin and Nirvanol from plasma

A 1-ml volume of plasma, 1 ml of 0.25 *M* sodium phosphate buffer (pH 6.5), 8 ml of chloroform, and internal standards (see section on calibration curves) were combined in a 13-ml ground glass stoppered centrifuge tube. The mixture was shaken for 10 min, centrifuged, and the aqueous layer removed by aspiration. The chloroform was then transferred to a clean centrifuge tube and evaporated to dryness on a Buchler rotary evaporator under water vacuum. The residue was dissolved in a mixture of 3 ml methanol and 2 ml of 0.25 *M* HCl. The acidified methanol mixture was shaken with 5 ml of hexane and then centrifuged. The aqueous layer was then transferred to a new tube and equilibrated with 8 ml of chloroform by shaking for 10 min. The chloroform layer was then removed and dried under water vacuum.

Perethylation of mephenytoin and Nirvanol

The perethylation reaction involved the addition of 200 μl of 2-butanone, 5 μl of 10 *N* KOH, and 50 μl of ethyl iodide to the extracted mephenytoin and Nirvanol. The centrifuge tube was capped, sealed with Parafilm and placed in a water-bath at 65° for 90 min. The tubes were then removed, cooled, and 2 ml of 0.25 *M* HCl and 8 ml of chloroform were added. The tubes were shaken for 5 min and then centrifuged. The chloroform layer was removed and dried under water vacuum.

The dried residue was then dissolved in 50—100 μl of methanol, and 1—2 μl of this mixture were injected into the gas chromatograph—mass spectrometer for analysis by the electron impact (EI) and, in some cases, chemical ionization (CI) modes.

Apparatus

The instruments used in this study were a Hewlett-Packard 5982 mass spectrometer with a combined EI/CI source in line with a Hewlett-Packard 5933 Dual Disc Data System. A Hewlett-Packard 5700 series gas chromatograph fitted with 1.2 m × 2 mm I.D. column packed with 2% OV-101 on 80—100 mesh Chromosorb W HP was used to separate the compounds. The column temperature was 175°, while the temperatures of the injection port and glass-lined single-stage jet separator were 250° and 300°, respectively. Helium was used as a carrier at a flow-rate of 30 ml/min, giving a source pressure of 4×10^{-6} Torr.

The mass spectrometer was operated with an electron current of 250 μA, electron energy of 70 eV, and an ion source temperature of 190°.

The selective ion monitoring was carried out in the EI mode, monitoring *m/e* 217 for MEPH, 230 for MCPH, and 231 for MPH and EPH. The dwell time for

each mass was 300 msec. The results obtained by the EI mode were verified by the CI mode, monitoring *m/e* 249 for MEPH and MPH, 259 for MCPH, and 261 for EPH.

Calibration curves

Mephenytoin and Nirvanol calibration curves were produced by adding various amounts of each drug and the appropriate internal standard to human plasma and then extracting these compounds as described above. The range of amounts of mephenytoin and Nirvanol added to plasma was chosen to encompass the unknowns.

RESULTS AND DISCUSSION

The mass spectra of the four perethylated hydantoins are shown in Fig. 2. In all cases the EI spectra show that elimination of the alkyl group from the 5-position appears to be the prime reaction under 70 eV. Both Nirvanol and MPH have the same base *m/e*, 231, whereas MEPH and MCPH have a base *m/e* of 217. In all cases, the relative abundance of molecular ion of the perethylated

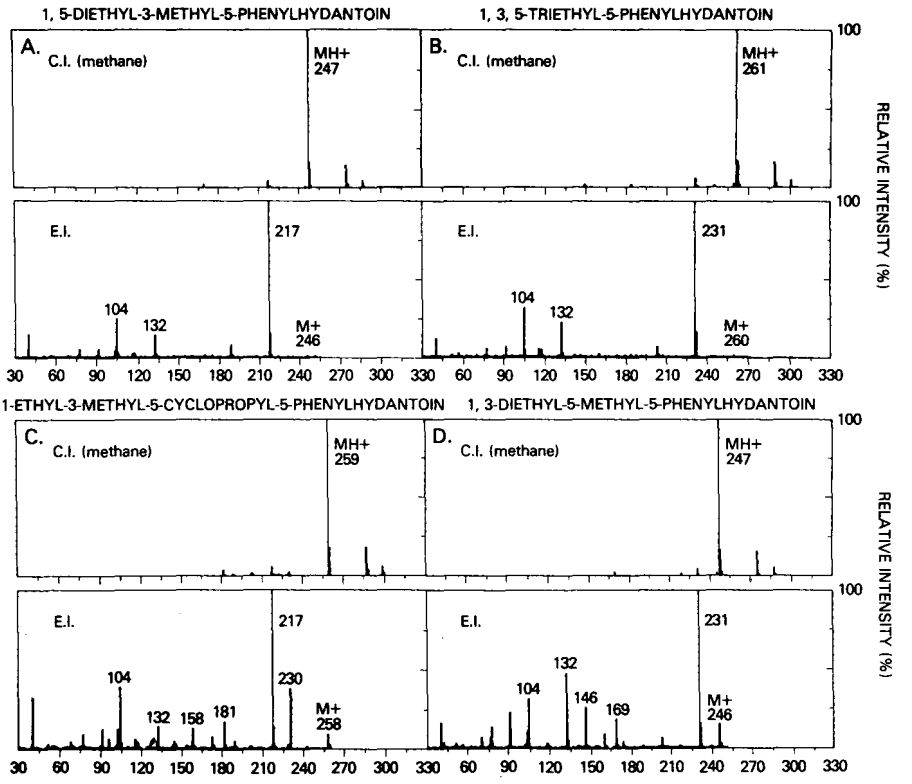


Fig. 2. Electron impact (EI) and chemical ionization (CI) (methane) mass spectra of the four perethylated hydantoins used in this study: (A) 1,5-diethyl-3-methyl-5-phenylhydantoin; (B) 1,3,5-triethyl-5-phenylhydantoin; (C) 1-ethyl-3-methyl-5-cyclopropyl-5-phenylhydantoin; (D) 1,3-diethyl-5-methyl-5-phenylhydantoin.

hydantoin is small. The CI spectra exhibit the characteristic quasimolecular ions, with little or no fragmentation of the molecule. The CI mode was applied to some of the initial samples to verify that the EI results were reproducible by a different means of quantification. In all cases the CI results were virtually identical to those of EI. Although the sensitivity of CI was greater than that of EI, the lower limits of sensitivity were not determined because the CI mode was used solely for verification.

The perethylation procedure appears to reach equilibrium in 60—90 min. Small amounts of N,N-dimethylated products are formed which may be the result of methyl iodide contamination. There also appear to be small peaks eluting before the perethylated mephenytoin and Nirvanol peaks which show the same base *m/e* as the perethylated compounds and have similar fragmentation patterns. They are most likely either N,O or O,O isomers.

The ethylation of the N-1 position appears to be a much slower reaction than ethylation of N-3. The progression of the reaction was followed to equilibrium by monitoring the appearance of the 1,3-diethylated Nirvanol. MPH was chosen as the internal standard for Nirvanol because it undergoes a two-step alkylation, similar to Nirvanol. Likewise, MCPH was chosen as the internal standard for mephenytoin because of its alkylation of the N-1 position. In fact, either internal standard could be used in the quantitation. In the case of MCPH, the base *m/e* of 217 was not used in the analysis because the patient received phenobarbital; phenobarbital elutes close to MCPH and has an interfering *m/e* of 217. In this case, an *m/e* of 230 was used instead.

The base and solvent appear also to play an important role in the alkylation of 5-alkyl-5-phenylhydantoins. Results similar to those found by De Sagher et al. [7] were observed. When base of weaker concentration (for example, 0.5 *N*) was used, substitution occurred only in the 3-position. A concentration of 5—10 *N* was found to be maximal. Bases other than KOH were tried with little or no success in forming the perethylated product. These were: 0.1 *M* tetrabutylammonium hydroxide, 2 *M* tetramethylammonium hydroxide, and 0.2 *M* trimethylphenylammonium hydroxide. Extractive alkylation, using ethyl iodide and tetrabutylammonium hydrogen sulfate as the counter-ion according to the method of De Silva and Bekersky [8] produced 3-ethyl substitution.

Solvents such as methylene chloride, benzene—methylene chloride (9:1), and hexane—isopropyl alcohol (9:1) resulted in similar monosubstitution, with small but variable amounts of the 1,3-diethyl derivative.

The EI—SIM calibration curve for mephenytoin was obtained by plotting the ratio of the area of mephenytoin (*m/e* 217) to the area of MCPH internal standard (*m/e* 230) against the concentration of mephenytoin in human plasma. The results were linear over the concentration range 0.01—3.0 μg/ml, with a representative curve giving an r^2 value of 0.9991 and a *Y*-intercept of —0.0125. The Nirvanol calibration curve was obtained by plotting the ratio of the area of Nirvanol (*m/e* 231) to the area of MPH internal standard (*m/e* 231) against the concentration of Nirvanol in human plasma. The results were also linear over the concentration range of 0.05—4.0 μg/ml, with a typical curve giving an r^2 value of 0.9994 and a *Y*-intercept of —0.0171. The calibration curve standards were injected into the gas chromatograph—mass spectrometer at various times during the analysis of the unknowns. The peak area ratios for these standards injected several times did not vary by more than 6%.

The recoveries of mephenytoin and Nirvanol by the use of this extraction procedure were found to be 90% for mephenytoin and 85% for Nirvanol and were consistent over the range analyzed.

Fig. 3 shows representative SIM fragmentograms (EI and CI) of an extract of plasma obtained from a patient receiving a single 50 mg oral dose of mephenytoin. This patient was also treated chronically with phenobarbital and primidone for seizure control. As mentioned above, the perethylated product of phenobarbital yields an *m/e* of 217 on EI mass spectrometry, which precludes the use of MCPH as an internal standard. Primidone, phenylethylmalonamide, phenytoin, and carbamazepine do not yield interfering fragments.

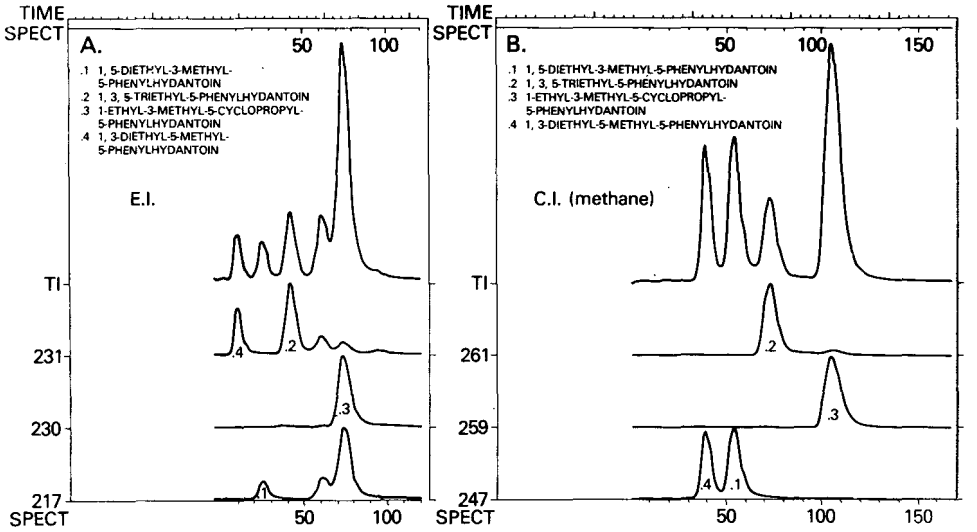


Fig. 3. Selective ion recordings of mephenytoin, Nirvanol, and their internal standards following extraction from plasma obtained from a patient receiving a single 50-mg oral dose of mephenytoin.

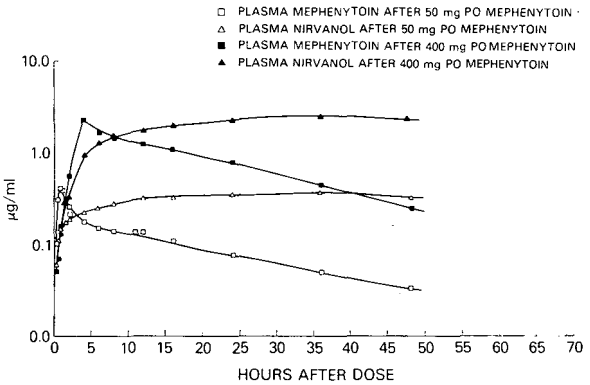


Fig. 4. The plasma concentrations of mephenytoin and Nirvanol in a patient after single oral doses of 50 mg and 400 mg of mephenytoin.

Fig. 4 shows the plasma levels of both mephenytoin and Nirvanol after the administration of 50 mg and 400 mg of mephenytoin in single oral doses. Mephenytoin was rapidly absorbed and disappeared from the plasma with a half-life of 12 h. Nirvanol slowly accumulated, reaching a maximum level in 24 h, and then slowly disappeared from the plasma. Nirvanol can be quantitated 144 h after the ingestion of a single 50-mg dose.

In conclusion, the gas chromatography—mass spectrometric selective ion monitoring technique can be used to measure submicrogram quantities of both mephenytoin and Nirvanol. Perethylation of these compounds produces stable derivatives and symmetrical chromatographic peaks. The choice of solvent and base is most important in determining the position of alkylation. The blood Nirvanol concentrations in patients on chronic mephenytoin therapy are high enough to be measured by standard chromatographic techniques because of the long half-life of Nirvanol. Selective ion monitoring analysis, however, can be most useful in determining the pharmacokinetic properties of mephenytoin and Nirvanol in epileptic patients.

ACKNOWLEDGEMENTS

The authors are indebted to R.J. Porter, M.D., and M.E. Newmark, M.D., for their help in obtaining patient samples.

REFERENCES

1 J.G. Millichap, in D.M. Woodbury, J.K. Penry and R.P. Schmidt (Editors), Antiepileptic Drugs, Raven Press, New York, 1972, pp. 275—281.
2 T.C. Butler, J. Pharmacol. Exp. Ther., 104 (1972) 299.
3 T.C. Butler, J. Pharmacol. Exp. Ther., 109 (1953) 340.
4 T.C. Butler and W. Waddell, J. Pharmacol. Exp. Ther., 110 (1954) 241.
5 H.J. Kupferberg and W. Yonekawa, Drug Metab. Dispos., 3 (1975) 26.
6 P. Friel and A.S. Troupin, Clin. Chem., 21 (1975) 751.
7 R. De Sagher, J. Pocius and J.-P. Thenot, J. Chromatogr., 156 (1978) 43.
8 J.A.F. de Silva and I. Bekersky, J. Chromatogr., 99 (1974) 447.

Journal of Chromatography, 163 (1979) 169—177
Biomedical Applications

CHROMBIO. 334

ISOLATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND QUANTITATION BY RADIOIMMUNOASSAY OF THERAPEUTIC CONCENTRATIONS OF DIGOXIN AND METABOLITES

HOWARD A. NELSON, SAMUEL V. LUCAS and THOMAS P. GIBSON

Veterans Administration Lakeside Hospital, General Medical Research Service, 333 East Huron Street, Chicago, Ill. 60611 (U.S.A.)

(Received October 31st, 1978)

SUMMARY

A method combining high-performance liquid chromatography for separation of digoxin from three of its metabolites with subsequent quantitation of each compound by radioimmunoassay is described. The metabolites are shown to interfere with the radioimmunoassay procedure thus providing the need for separation prior to assay.

INTRODUCTION

Although radioimmunoassay (RIA) is by far the most commonly used method to quantitate therapeutic levels (0.5—2.5 ng/ml) of digoxin in plasma, antibodies used in this assay may not be specific for the parent drug. Stoll et al. [1] have shown that three digoxin metabolites: digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, and digoxigenin all cross-react with the digoxin "specific" antibody. Therefore pharmacokinetic studies based on the use of a nonspecific assay are open to question [2] especially if the studies were done in clinical settings, such as end-stage renal failure, where the accumulation of digoxin metabolites is possible.

It has been shown that the pharmacokinetics of digoxin are altered in human renal failure wherein the rate of digoxin elimination as well as the volume of distribution are reduced [2]. Since approximately 75% or more of a dose of digoxin is eliminated unchanged in the urine of a patient with normal renal function [3] the decrease in elimination in renal failure is to be expected. However, the surprising decrease in the volume of distribution from 10 l/kg in normal [4] to 5 l/kg in renal failure patients [2] is as yet unexplained and may well be an artifact of the RIA caused by the accumulation of digoxin metabolites which will react with the digoxin RIA antibody. Therefore, before a study of the pharmacokinetics of digoxin in renal failure could be undertaken

it was necessary to develop an analytical method specific for digoxin. This paper describes a high-performance liquid chromatographic (HPLC) procedure to separate digoxin from its metabolites with subsequent assay of the individual compounds by ^{125}I RIA.

EXPERIMENTAL

Materials

All solvents used in the extraction or chromatography were Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) "distilled in glass" grade. Water was distilled in an all-glass still. Digoxin, digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, and digoxigenin were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind., U.S.A.). The RIA was performed using the Ab-TRAC (T.M.) Digoxin Solid Phase Radioimmunoassay Kit (^{125}I) manufactured by Becton Dickinson Immunodiagnostic (Orangeburg, N.Y., U.S.A.). Tritium-labelled digoxin was obtained from New England Nuclear (Boston, Mass., U.S.A.) and was purified before use by HPLC under the conditions described later. The scintillation solution used was Complete Counting Cocktail 3a70B manufactured by Research Products International (Elk Grove Village, Ill., U.S.A.). Tritium-labelled metabolites were prepared by hydrolysis of labelled digoxin with 1 *N* hydrochloric acid for 10 min at room temperature. The reaction mixture was then neutralized with sodium hydroxide and extracted into dichloromethane. The dichloromethane was evaporated to dryness under a gentle stream of nitrogen in a water bath at 37° and the residue redissolved in 0.2 ml of the chromatographic mobile phase. Separation of these hydrolysis products was accomplished by HPLC using the conditions described later. Half-minute elution fractions were collected and the radioactivity of an aliquot of each was measured by scintillation counting. Retention volumes of the tritium-labelled components were then compared to a chromatogram of the authentic (unlabelled) metabolites and the fractions from the hydrolysis products corresponding to the three metabolites were saved.

Plasma extraction

Approximately 5000 dpm of chromatographically pure [^{3}H]digoxin in 0.02 ml buffer was added to 1 ml of plasma in a 16 × 150 mm PTFE-lined screw-cap tube, vortexed, and allowed to stand for 5 min. The same amount of labelled digoxin was added to a scintillation vial for recovery monitoring.

The sample was shaken for 5 min with 12 ml of dichloromethane, centrifuged 15 min and the aqueous layer aspirated. The dichloromethane was then shaken with 1 ml of 0.1 *N* sodium hydroxide solution for 1 min, centrifuged for 15 min and the aqueous layer aspirated. After drying with about 0.5 g of anhydrous sodium sulfate, the dichloromethane was evaporated to dryness under a gentle stream of nitrogen at 37°. The residue was dissolved in 0.2 ml of HPLC mobile phase with vigorous vortexing.

Chromatography

The HPLC system consisted of two Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A pumps, a Model 660 solvent programmer, A Model U6-K injec-

tor and a Model 440 absorbance detector operating at 254 nm. Samples were chromatographed on a μBondapak C_{18} (Waters Assoc.) column (30 cm × 3.9 mm I.D.) with a 7-min gradient from 53% to 62% methanol in water. The fractions containing the digoxin and the metabolites were collected for further assay by ^{125}I RIA and scintillation counting and were evaporated to dryness in a rotary evaporator.

Radioimmunoassay

The dried down fractions from the chromatograph were reconstituted in 1.0 ml of the RIA kit buffer which had been diluted to 400 ml with distilled water containing 2% propylene glycol and 5% blank plasma. The propylene glycol and blank plasma are a modification of the RIA kit procedure. The plasma standards supplied with the kit were not used. Instead, standards were prepared in the modified buffer from weighed quantities of digoxin and the metabolites. The standards included a blank, 0.5, 1.0, 1.67, 2.5, 3.33, and 5.0 ng/ml. All samples and the standards were analyzed in duplicate. A blank plasma was also extracted and run through the procedure. The standard curve was plotted on logit—log paper to give the best straight line [5]. The abscissa was the log of the concentration and the ordinate was the logit of percent of ^{125}I trace level binding.

Recovery monitoring

The recovery of digoxin varied from 54% to 78% through the procedure prior to RIA. Therefore, an average recovery cannot be assumed and tritium-labelled digoxin was used to measure recovery through the procedure.

The amount of tritium-labelled digoxin added to the plasma sample as an internal standard was equal to about 5000 dpm in 0.02 ml. With the dilutions used this is about 0.1 ng and this added amount of digoxin must be taken into account when calculating the initial digoxin concentration of the sample. This correction is shown in the next section.

A 0.4-ml portion of the 1.0 ml reconstituted in buffer fraction from the liquid chromatograph was transferred to a scintillation vial and 10 ml of scintillation mixture added. This was counted for 20 min in a liquid scintillation counter along with the vials containing the total counts added at the beginning of the extraction. Added to the total counts vials was 0.38 ml of buffer to equalize any quenching by having the same volume of buffer in all samples. The calculation of the recovery was done as follows.

$$\text{Percent recovery} = \frac{\text{Counts in sample}/0.4}{\text{Total counts added}}$$

RESULTS AND DISCUSSION

Quantitation

RIA was performed on the samples taken off the liquid chromatograph with the standards prepared in buffer. Fig. 1 shows a typical standard curve for digoxin. The concentrations read off the standard curve were manipulated as

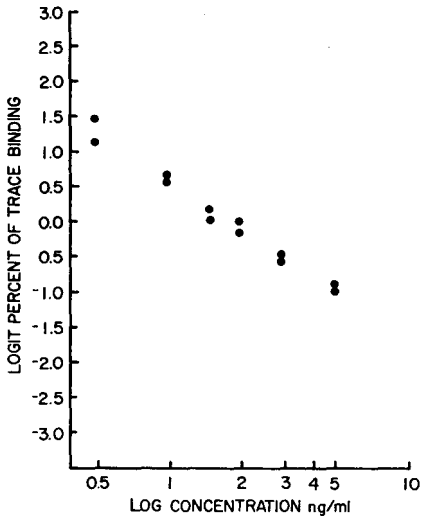


Fig. 1. Radioimmunoassay standard curve for digoxin.

follows to take into account the recovery loss, the amount of labelled compound added as recovery tracer and the blank reading.

$$C_i = \frac{\frac{C_f - C_b}{R} V_r - C_t V_t}{V_e}$$

where C_i = initial unknown concentration of the sample; C_f = final concentration from RIA; C_b = concentration of the blank extract; R = recovery of labelled tracer through procedure; V_r = volume used to reconstitute dried sample; C_t = concentration of labelled recovery tracer; V_t = volume of recovery tracer added; V_e = volume of sample extracted.

Chromatography

Fig. 2 shows a chromatogram of the separation of a mixture containing digoxin and the three metabolites. The sample contained 30 μg each of digoxin and the mono- and bisdigitoxoside and 10 μg of digoxigenin. The extinction coefficients of digoxin and the metabolites at the wavelength used (254 nm) prevent direct photometric detection in the therapeutic range (0.5—2.5 ng/ml). While other workers have demonstrated a 40-fold increase in sensitivity at 220 nm instead of 254 nm [6] one still would need at least a further 5—10-fold sensitivity improvement to measure digoxin and the metabolites directly. However, with the baseline separation achieved by this HPLC method the fractions containing each of the compounds can be collected and assayed by RIA which does have sufficient sensitivity.

Since pharmacological concentrations of digoxin and metabolites cannot be detected photometrically some other method must be used to determine the retention volumes containing these compounds. Chromatography with micro-

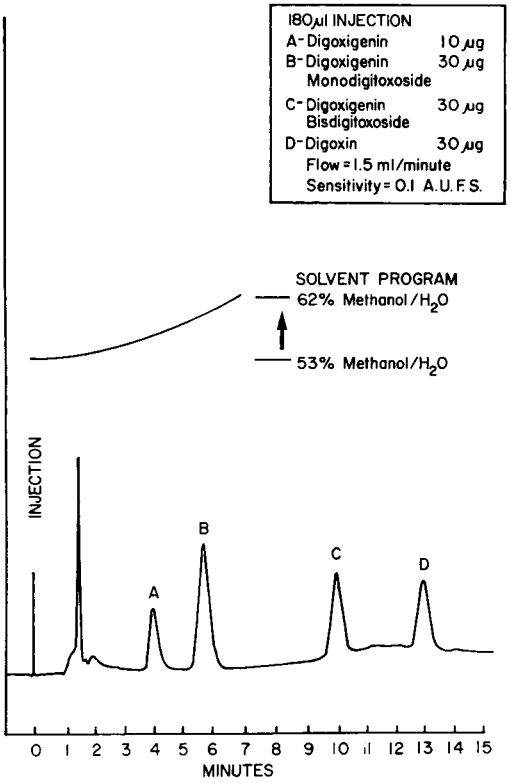


Fig. 2. Chromatogram of digoxigenin (A), digoxigenin monodigitoxoside (B), digoxigenin bisdigitoxoside (C), and digoxin (D). The chromatographic conditions are described in the text.

gram quantities as in Fig. 2 is helpful in initially determining the retention volumes with various solvent systems. However, this cannot be used prior to injecting extracts from plasma. We have found that after injection of microgram quantities of digoxin and metabolites for UV detection there was always a small but unavoidable carryover into subsequent injections which, although too small to be detectable photometrically, could easily interfere with the quantitation by RIA. This result is not unexpected since a 30-µg injection is ten thousand times higher than the nanogram levels normally achieved clinically and detectable by RIA thus carryover in the injection device at this level would not be surprising. Use of the tritium-labelled compounds works well to determine the retention volumes. The fractions can be collected coming off the column in scintillation vials, counted and retention volumes of the compounds determined.

Fig. 3 shows a typical chromatogram of a plasma extract. The retention volumes were determined in an earlier run. The peaks are endogenous material in the plasma.

Recoveries

The addition of 2% propylene glycol and 5% blank plasma to the RIA buffer

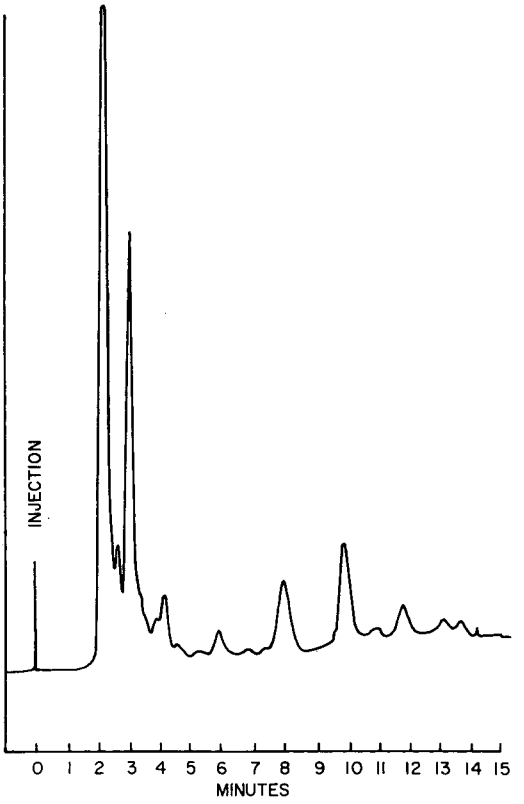


Fig. 3. Chromatogram of extracted plasma.

was necessary to obtain complete dissolution of the HPLC fractions after evaporating to dryness.

The ratio of the recoveries of the metabolites relative to digoxin (Table I) was determined as follows: Tritium-labelled digoxin and each of the three tritium-labelled metabolites were added to five plasma samples. The plasma samples were extracted and injected onto the liquid chromatograph. The eluted fractions containing the digoxin and the metabolites were collected and scintillation counted. The recovery of each compound was determined by the ratio of the amount of labelled compound added to the amount found in the eluted fraction.

Repeated digoxin assay on several days

Precision studies were carried out over several days by repeated analyses of plasma samples with added digoxin. The results are shown in Table II. Adversely affecting the precision of this assay is the necessity of adjusting the result read from the RIA standard curve for the losses in recovery as determined with the tritium-labelled tracer. For example, a sample with 2.0 ng/ml and 70% recovery should give an RIA value of 1.4 ng/ml. The error associated with the RIA result is about ± 0.1 ng/ml and the error in the tracer recovery result is ± 2%. Thus the calculated result could be between 1.8 and 2.2 ng/ml. As the

TABLE I

OVERALL RECOVERY (EXTRACTION AND CHROMATOGRAPHY) OF DIGOXIN AND METABOLITES

Compound	Trial No.	Recovery (%)	Metabolite/digoxin recovery ratio	Mean, S.D.
Digoxigenin	1	73.9	0.99	0.97, 0.03
	2	67.5	0.91	
	3	72.3	0.99	
	4	69.2	0.99	
	5	72.5	0.97	
Digoxigenin monodigitoxoside	1	68.1	0.91	0.89, 0.03
	2	64.8	0.87	
	3	62.2	0.85	
	4	62.5	0.89	
	5	68.5	0.91	
Digoxigenin bisdigitoxoside	1	70.9	0.95	0.97, 0.02
	2	71.0	0.95	
	3	72.3	0.99	
	4	70.4	1.00	
	5	73.4	0.98	
Digoxin	1	74.9		
	2	74.5		
	3	73.2		
	4	70.2		
	5	74.9		

TABLE II

PRECISION OF REPEATED DIGOXIN ASSAYS

Digoxin (ng/ml)	*n*	S.D. (ng/ml)	Coefficient of variation (%)
0.6	8	0.12	21.1
1.1	17	0.18	15.9
2.4	15	0.17	7.3
3.4	18	0.33	9.8
5.7	7	0.21	4.1

actual amount of digoxin or the recovery goes down the effect on the error increases. Analyses with lower than 50% recovery should be repeated. More than 1 ml of plasma can be extracted for samples with low levels of digoxin.

Assay of digoxin together with metabolites

Stoll et al. [1] found that digoxigenin monodigitoxoside and digoxigenin bisdigitoxoside were capable of binding to the antibody of a commercially available kit to nearly the same degree as digoxin itself. Digoxigenin had somewhat less affinity.

The results with this RIA procedure were the same. Figs. 4—6, show the

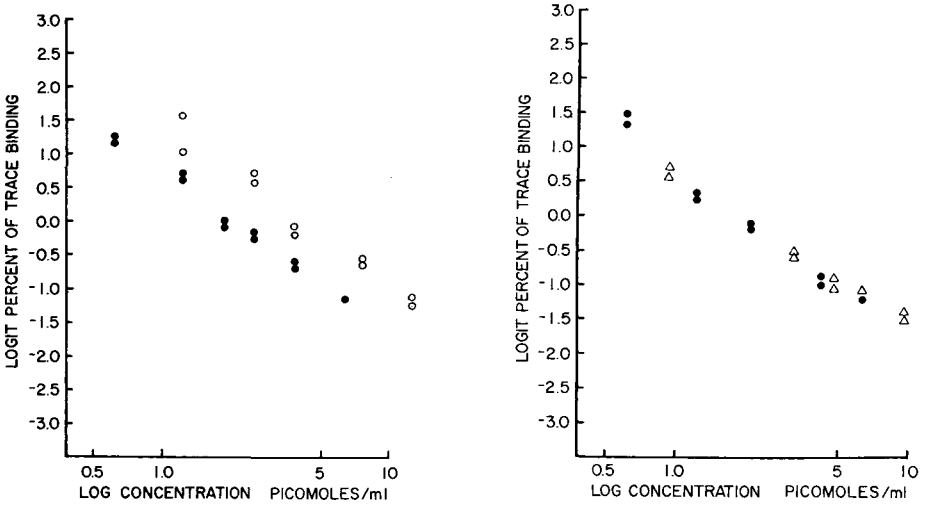


Fig. 4. Radioimmunoassay standard curve for digoxin (•) and digoxigenin (○) run simultaneously.

Fig. 5. Radioimmunoassay standard curves for digoxin (•) and digoxigenin monodigitoxoside (△) run simultaneously.

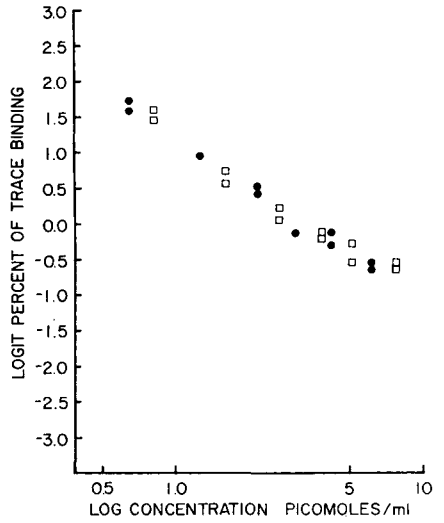


Fig. 6. Radioimmunoassay standard curves for digoxin (•) and digoxigenin bisdigitoxoside (□) run simultaneously.

molar RIA standard curves of digoxin compared with the three metabolites. The digoxin and metabolite standards in each case were assayed at the same time.

The effectiveness of the HPLC separation of digoxin from the three metabolites and their subsequent quantitation by RIA is shown in Table III. For each metabolite, three plasma samples (each in duplicate) were extracted, chromato-

TABLE III

ASSAY OF DIGOXIN TOGETHER WITH METABOLITES IN PLASMA SAMPLES

The concentrations of digoxin and metabolite in the third column were equal to those in the first and second columns, respectively.

Digoxin only (pmole)	Metabolite only (pmole)	Digoxin and metabolite (pmole)
1.17	2.97 (digoxigenin)	4.13
5.01	10.61 (Monodigitoxoside)	15.85
1.28	1.64 (Bisdigitoxoside)	3.29

graphed and assayed by RIA. The first two columns show the amounts found in the sample containing digoxin or metabolite only. The third column shows the sum of the number of pmoles of both digoxin and metabolite found in the sample containing both. This sum should be equal to the sum of the amounts in the first two columns, and in the three cases are nearly equal.

CONCLUSION

This study demonstrated that the presence of three of the metabolites of digoxin can interfere with the RIA of digoxin and that reversed-phase partition HPLC is suitable for separating the four compounds prior to the measurement of each by RIA.

ACKNOWLEDGEMENTS

Supported by the Medical Research Administration of the Veterans Administration; a grant from the Chicago Heart Association; and the Clinical Research Center of Northwestern University, RR-48, Division of Research Resources, National Institutes of Health.

REFERENCES

1 R.G. Stoll, M.S. Christensen, E. Sakmar and J.G. Wagner, Res. Comm. Chem. Pathol. Pharmacol., 4 (1972) 503.
2 J.R. Koup, W.J. Jusko, C.M. Elwood and R.K. Kohli, Clin. Pharmacol. Ther., 18 (1975) 9.
3 J.R. Koup, W.J. Greenblatt, W.J. Jusko, J.W. Smith and J. Koch-Wester, J. Pharmacokin. Biopharm., 3 (1975) 181.
4 W.G. Kramer, R.P. Lewis, T.G. Cobb, W.G. Forester, J.A. Visconti, L.A. Wauke, H.G. Boxenbaum and R.H. Reuning, J. Pharmacokin. Biopharm., 2 (1974) 299.
5 D. Rodbard, W. Bridson and P.L. Rayford, J. Lab. Clin. Med., 74 (1969) 770.
6 M.C. Castle, J. Chromatogr., 115 (1975) 437.

Journal of Chromatography, 163 (1979) 179—185
Biomedical Applications

CHROMBIO. 335

DETERMINATION OF PLASMA CONCENTRATIONS OF DAPSONE, MONOACETYL DAPSONE AND PYRIMETHAMINE IN HUMAN SUBJECTS DOSED WITH MALOPRIM

C.R. JONES and S.M. OVENELL

Department of Drug Metabolism, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS (Great Britain)

(First received October 27th, 1978; revised manuscript received February 26th, 1979)

SUMMARY

A high-performance liquid chromatographic method was developed to enable dapsone, monoacetyl dapsone and pyrimethamine to be measured simultaneously in plasma samples from volunteers in England and Malaysia who had been dosed with Maloprim. Mean half-lives of 25 and 80 h were calculated for dapsone and pyrimethamine, respectively, but there was wide individual variation. All subjects were found to be classifiable as "slow acetylators".

INTRODUCTION

Maloprim* is a product which contains pyrimethamine and dapsone; these together act synergistically in suppressing malaria in subjects who are exposed to this disease. In order to monitor a course of treatment a method was developed using high-performance liquid chromatography in which these substances were measured together with the expected monoacetylated metabolite of dapsone in the plasma of volunteers either working at these Laboratories or in Kuala Lumpur who had received Maloprim.

MATERIALS AND METHODS

The following substances were used as reference compounds in developing the analytical method. Pyrimethamine [2,4-diamino-5-(4-chlorophenyl)-6-ethylpyrimidine]; dapsone (DDS) (4,4′-diaminodiphenyl sulphone); monoacetyl dapsone (MADDS) (4-N-acetylamino-4′-aminodiphenyl sulphone); and metoprine [2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine].

*Maloprim is a licenced product of the Wellcome Foundation Ltd.

Volunteer studies

Three members of the staff at Wellcome Research Laboratories, Beckenham who were working with mosquito transmitted *Plasmodium knowlesi* received Maloprim as protection against malaria. Each week they received a tablet containing 12.5 mg pyrimethamine and 100 mg dapsone; blood samples were taken at 0.5, 1, 2, 4, 6, 8 and 24 h after the first dose and then at the same time of day as the original dosing each day for 7 days.

Plasma samples from nine volunteers who received a single tablet of Maloprim at the Institute for Medical Research in Kuala Lumpur were also assayed. Samples were taken at 24 h after the dose and then once daily at the same time for a further 6 days. The volunteers were all male Indians aged between 18 and 30 and living as they were in an area of continuous malaria transmission can be regarded as semi-immune.

Extraction procedure

Duplicate amounts of 2 ml from each plasma sample were diluted with an equal volume of water, made alkaline with sodium hydroxide and mechanically mixed with 2 portions of 4 ml of 1,2-dichloroethane. After centrifugation the pooled extracts were evaporated under a stream of nitrogen gas, the residue was dissolved in 50 μl of the same solvent and transferred to a small capped vial ready for high-performance liquid chromatography.

Metoprine, an analogue of pyrimethamine, was routinely added at 2 μg/ml to all the plasma samples as an internal standard. It was shown to extract identically to pyrimethamine under the conditions used, the overall recovery being close to 80%. DDS and MADDS were found to extract almost quantitatively, so the internal standard served to make allowance for any variations occasioned by, for instance, evaporation of solvent.

HPLC assay method

A Perkin Elmer Model 1210 liquid chromatograph fitted with a fixed-wavelength (254 nm) UV absorbance detector was used and the concentration of the components determined by comparison of the peak areas with that of the internal standard. A Hewlett-Packard Model 3352 data processor was used to derive the peak areas and, as the response was not linear, a further correction based upon comparison with extracts from plasma samples "spiked" with DDS, MADDS and pyrimethamine at known concentrations was made. Because the detector was somewhat insensitive and the concentrations of the measured substances were low, more than half the extract had usually to be injected.

Using di-isopropyl ether—methanol—21% aqueous ammonium hydroxide (96:4:0.1) at a flow-rate of 2 ml/min through a 100 × 4.6 mm I.D. stainless-steel column packed with 5-μm spherical silica (Spherisorb S5W; Phase Separations, Queensferry, Great Britain), DDS, pyrimethamine, metoprine and MADDS were successfully separated in about 7 min. A representative chromatogram is shown in Fig. 1.

The method proved valid over a range from 0 to 4 μg/ml; the coefficient of variation between duplicates varied with concentration and ranged from 5.0% for DDS at levels above 500 ng/ml to 19.7% for MADDS at levels below

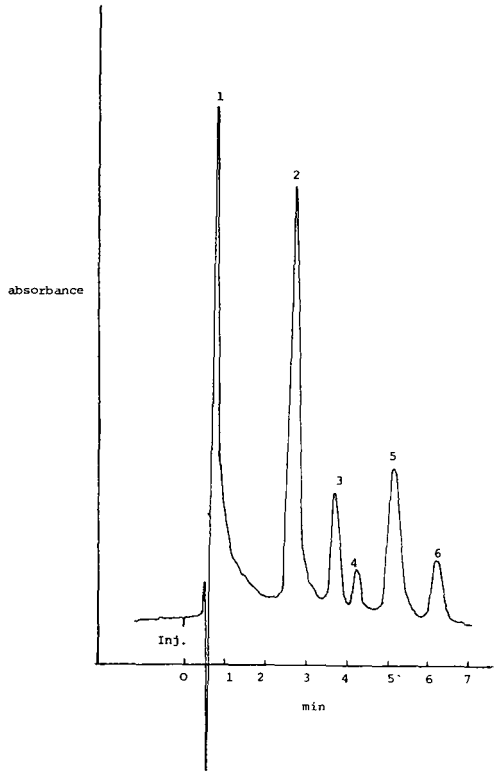


Fig. 1. Representative chromatogram of plasma extract. Extract from human plasma, spiked before extraction with internal standard. Conditions as in text. Peaks: 1, solvent front; 2, DDS; 3, pyrimethamine; 4, unknown plasma constituent; 5, internal standard; 6, MADDS.

50 ng/ml. The overall mean coefficient of variation was 11.3% and the lower limit of detection (i.e. signal-to-noise ratio = 2) was about 5 ng injected for pyrimethamine and 2.5 ng for the other substances.

Half-lives

Half-lives were calculated from semilogarithmic plots of plasma concentrations against time, the method of least squares being employed for fitting a straight line to the values.

RESULTS

Individual plasma concentrations for the Beckenham samples are shown in Table I and the mean values of the Malaysian samples in Fig. 2. Individual variation in plasma half-lives was considerable (Table II), particularly those of pyrimethamine. The mean value for all volunteers for pyrimethamine was 80 h (range 35 to 175 h) and for DDS was 25 h (range 16 to 38 h).

Highest levels as seen from the Beckenham samples were obtained at ca. 4 to 8 h for DDS and 8 to 24 h for pyrimethamine, although in some subjects both compounds showed an earlier less pronounced peak.

TABLE I

PLASMA LEVELS OF DDS, MADDS AND PYRIMETHAMINE (ng/ml) IN BECKENHAM SUBJECTS AT DIFFERENT TIMES AFTER DOSING WITH MALOPRIM

Time after 1st dose	DDS			MADDS			Pyrimethamine		
	W.H.G.R.	D.D.	M.P.	W.H.G.R.	D.D.	M.P.	W.H.G.R.	D.D.	M.P.
0.5 h	30	710	650	0	430	200	0	20	31
1 h	76	1300	1250	4	580	350	7	53	100
2 h	250	1310	1460	29	460	360	15	55	85
4 h	880	1440	1340	140	450	350	120	82	74
6 h	1150	1170	1210	170	330	280	16	69	66
8 h	1360	1140	1480	270	330	360	68	51	100
Day 1	1080	710	620	350	170	180	150	56	68
Day 2	550	440	240	120	180	77	75	64	75
Day 3	280	180	81	44	85	30	31	57	59
Day 4	110	82	32	31	61	10	24	59	69
Day 5	66	26	10	26	22	0	40	51	31
Day 6	38	21	0	15	18	0	20	52	31
Day 7	16	10	0	8	7	0	21	44	26
Second dose Day 8	910	910	530	130	320	190	56	100	93

TABLE II

PLASMA HALF-LIVES (h)

Subjects		DDS		Pyrimethamine	
		$t_{½}$	S.D.	$t_{½}$	S.D.
Malaysian	A	23.5	0.05	73.0	0.70
	B	21.1	0.04	67.7	0.67
	C	20.6	0.07	82.1	1.69
	D	29.5	0.04	55.4	0.05
	E	38.2	0.17	174	1.14
	F	20.9	0.05	100	0.41
	H	34.3	0.03	102	0.81
	J	22.3	0.08	54.0	0.34
	K	31.2	0.11	34.8	0.29
Beckenham	W.H.G.R.	24.0	0.02	51.6	1.43
	D.D.	22.3	0.05	N.V.*	N.V.*
	M.P.	16.3	0.01	56.9	0.97

*N.V. = not valid.

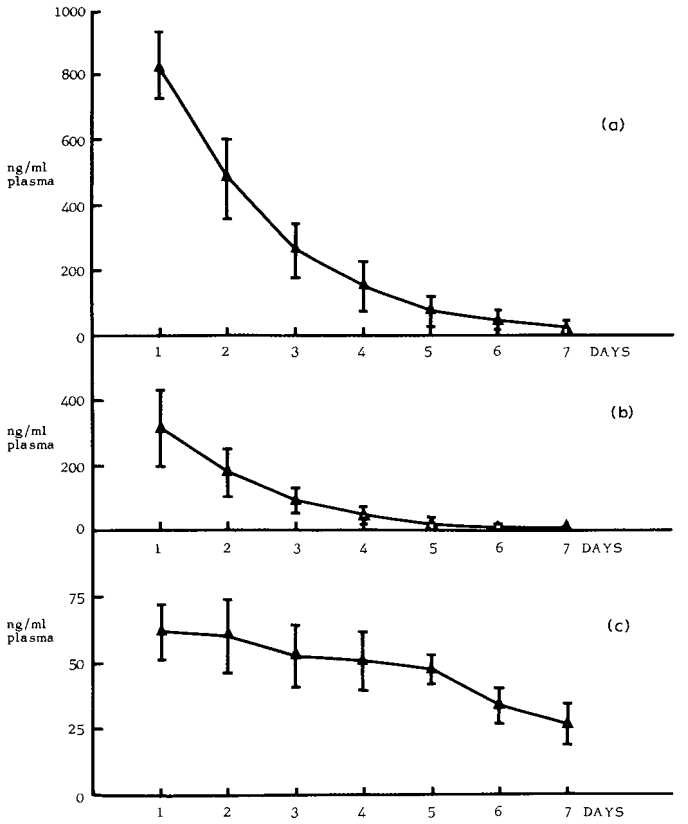


Fig. 2. Plasma concentrations measured by high-performance liquid chromatography in Malaysian subjects. Mean of 9 individuals ± 1 standard deviation. (a) Dapsone, (b) N-acetyl dapsone, (c) pyrimethamine.

By comparing the MADDS concentrations with those of DDS the acetylator status of each individual could be assessed.

DISCUSSION

Half-lives

Whereas semilogarithmic plots of plasma concentration against time gave an almost linear regression and indicated a close approximation to first order kinetics for DDS, this was not true for pyrimethamine. Whether or not the pharmacokinetics were genuinely non-linear could best be decided from a larger number of subjects. Plasma half-lives for both drugs calculated from this study, despite a wide individual variation, showed reasonable agreement with those of other workers [1—5].

It was possible to detect individual variation in rates of absorption in Beckenham volunteers (Table I). One volunteer (WHGR) apparently absorbed both constituents from the first dose more slowly than did the others, but these differences in plasma concentrations were largely gone by day 2.

The second dose resulted in a return to approximately the same plasma values at 24 h as after the first.

Acetylated metabolites of DDS

Formation of several metabolites of DDS have been reported. N-Glucuronidation [6], N-sulphation [7], N-hydroxylation [8] and N-acetylation [9] have been described. Of these, a simple extract of plasma, as used here, is only likely to show any mono- and diacetylated compounds which may be present. Peters et al. [10] stated that DADDS is not detectable in human plasma after dosing with DDS. By comparison with authentic DADDS we were able to find traces of this metabolite in the plasma of these volunteers, but the concentrations were so low as to be ignored.

Man exhibits genetic polymorphism in the metabolic acetylation of certain compounds including DDS and ratios of concentrations of MADDS to DDS in human plasma are sufficient to assign individuals to appropriate acetylator categories [9].

In this study the fastest acetylator had a MADDS value of 32% of total sulphone measured at 24 h and the mean value for all subjects was 22% (S.D. = 6.2%). Gelber et al. [9] defined fast acetylators as having a corresponding value of at least 40% with a mean above 50%. Hence all subjects in this study both from Beckenham and Malaysia can be classified as slow acetylators.

Comments on the analytical method

DDS and MADDS can be measured by fluorescence techniques [3, 4] and when a fluorescence detector is coupled to a high-performance liquid chromatograph a highly sensitive method is obtained [12, 13]. However, pyrimethamine is poorly fluorescent in solution and a method was required here which would adequately measure the plasma concentrations of all compounds simultaneously.

Levels of pyrimethamine in biological samples have long proved difficult to measure because of the small doses given and the necessity to measure the drug at long periods after dosing. Following thin-layer chromatography De Angelis et al. [11] used a photometric plate scanner to measure this compound but the possibility of naturally occurring compounds co-chromatographing with pyrimethamine is much higher for thin-layer chromatography than for high-performance liquid chromatography.

Using the liquid chromatographic method the minimum concentrations which could be reliably measured were approximately 10 ng/ml for pyrimethamine and 5 ng/ml for DDS and MADDS. Columns of higher efficiency and more sensitive detectors could further improve this technique for such assays.

A gas—liquid chromatographic method for pyrimethamine has been reported [14]. An improved method also based upon gas—liquid chromatography is under development in these laboratories to exploit the potentially greater sensitivity for measurement of pyrimethamine.

ACKNOWLEDGEMENTS

Our grateful thanks are due to Mrs. V. Lees and Mrs. E. Norrie for technical assistance, to Drs. R.H. Nimmo-Smith and B.C. Weatherley for helpful discussions and to Drs. D.B.A. Hutchinson and J.T. Ponnampalam who arranged the Malaysian volunteer studies and supplied the samples.

REFERENCES

1 C.C. Smith and J. Ihrig, Amer. J. Trop. Med. Hyg., 8 (1959) 60.
2 D.R. Stickney, W.S. Simmons, R.L. De Angelis, R.W. Runelles and C.A. Nichol, Proc. Amer. Ass. Cancer Res., 14 (1973) 52.
3 A.J. Glazko, W.A. Dill, R.G. Montalbo and E.L. Holmes, Amer. J. Trop. Med. Hyg., 17 (1968) 465.
4 G.A. Ellard and P.T. Gammon, Int. J. Lepr., 37 (1969) 398.
5 J.H. Peters, G.R. Gordon, D.C. Ghoul, J.G. Tolentino, G.P. Walsh and L. Levy, Amer. J. Trop. Med. Hyg., 21 (1972) 450.
6 S.R.M. Bushby, Int. J. Lepr., 35 (1967) 572.
7 S. Tsutsumi, Chem. Pharm. Bull., 9 (1961) 432.
8 Z.H. Israili, S.A. Cucinell, J. Vaught, E. Davis, J.M. Lesser and P.G. Dayton, J. Pharmacol. Exp. Ther., 187 (1973) 138.
9 R. Gelber, J.H. Peters, G.R. Gordon, A.J. Glazko and L. Levy, Clin. Pharmacol. Ther., 12 (1971) 225.
10 J.H. Peters, G.R. Gordon and W.T. Colwell, J. Lab. Clin. Med., 76 (1970) 338.
11 R.L. DeAngelis, W.S. Simmons and C.A. Nichol, J. Chromatogr., 106 (1975) 41.
12 C.A. Mannan, G.J. Krol and B.T. Kho, J. Pharm. Sci., 66 (1977) 1618.
13 J. Vansant, R.L. Woosley, J.T. John and J.S. Sergent, Arthritis Rheum., 21 (1978) 192.
14 P.C. Cala, N.R. Trenner, R.P. Buhs, G.V. Downing, Jr., J.L. Smith and W.J.A. VandenHeuvel, J. Agr. Food Chem., 20 (1972) 337.

Journal of Chromatography, 163 (1979) 187—193
Biomedical Applications

CHROMBIO. 326

SIMULTANEOUS DETERMINATION OF THE ANTICONVULSANTS, CINROMIDE (3-BROMO-N-ETHYLCINNAMAMIDE), 3-BROMOCINNAMAMIDE, AND CARBAMAZEPINE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

ROBERT J. PERCHALSKI*, JOSEPH BRUNI, B.J. WILDER and L.J. WILLMORE

Research and Neurology Services, Veterans Administration Medical Center, and Department of Neurology, College of Medicine, University of Florida, Gainesville, Fla. (U.S.A.)

(First received November 23rd, 1978; revised manuscript received February 19th, 1979)

SUMMARY

A high-performance liquid chromatographic method is described for monitoring plasma concentrations of cinromide (3-bromo-N-ethylcinnamamide) and its de-ethylated metabolite. Carbamazepine levels can be easily measured by the same technique. The N-isopropyl analogue of cinromide is used as internal standard, and all compounds are easily separated on a reversed-phase column operated at 55° with a small-diameter pre-column maintained at the same temperature. The extraction is rapid and generally applicable to plasma and urine samples that are to be analyzed by reversed-phase chromatography. Short- and long-term reproducibility studies show less than 4% relative standard deviation for replicate determinations for all drugs. Limits of quantitation are 10—20 ng/ml with an internal standard concentration of 3 μg/ml. Another metabolite of cinromide, 3-bromocinnamic acid, which may have some anticonvulsant effect, can be analyzed simultaneously by buffering the mobile phase and adding an ion-pairing reagent.

INTRODUCTION

Cinromide (3-bromo-N-ethylcinnamamide) (I) and its de-ethylated metabolite, 3-bromocinnamamide (II), exhibit strong protection against maximal electroshock convulsions, pentylenetetrazole induced convulsion and psychomotor seizures in rodents [1]. The drug is currently undergoing clinical testing for use as a broad-spectrum anticonvulsant.

During the development of a method for the determination of I and II in the plasma of epileptic patients, both gas—liquid chromatography (GLC) and high-performance liquid chromatography (HPLC) were investigated. Flame ioniza-

*To whom correspondence should be addressed at the Research Service (151), Veterans Administration Medical Center, Gainesville, Fla. 32602, U.S.A.

tion GLC was excluded because of poor sensitivity. Adequate peak shape and resolution were achieved, however, with various polyester stationary phases. Because of the sensitivity required and the high absorptivity of the compounds of interest, reversed-phase HPLC with UV absorption detection was selected as the method of choice. Since carbamazepine (III) is a commonly used anticonvulsant and is functionally and chromatographically similar to II, a method was developed that allows simultaneous determination of I, II and III.

EXPERIMENTAL

Reagents

All chemicals were analytical reagent grade. Acetonitrile and iso-octane were used as purchased (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.). Water was purified for use by passing it through an organic absorbing ion-exchange system (Bion Exchange System D, Pierce, Rockford, Ill., U.S.A.). The internal standard (3-bromo-N-isopropyl cinnamamide [IV]), I, II and 3-bromocinnamic acid were used as received (Burroughs-Wellcome, Research Triangle Park, N.C., U.S.A.). Carbamazepine was recrystallized from tablets as previously described [2].

The drug standard stock solution was made by dissolving 3 mg, 7 mg, and 12 mg of I, II and III, respectively, in absolute ethanol and diluting to 25 ml. The working drug standard solution was made daily by diluting 0.200 ml of stock solution to 2.00 ml with acetonitrile.

The internal standard stock solution was made by dissolving 1.9 mg of IV in absolute ethanol and diluting to 25 ml. The working internal standard was made fresh at least every other day by diluting 1.00 ml of stock solution to 25 ml with aqueous 0.1 *M* ascorbic acid. This solution has been used previously to increase the stability of an aqueous internal standard [2].

Apparatus

The liquid chromatograph consisted of a reciprocating piston pump with a pressurized tube pulse dampener and a fixed-wavelength UV absorbance detector (Laboratory Data Control, Riviera Beach, Fla, U.S.A.), operated at 280 nm with a static nitrogen reference. The column (250 mm × 4.6 mm I.D.) was packed with a 10-μm microparticulate C_{18} reversed phase (Reeve-Angel, Liquid Chromatography Division, Clifton, N.J. U.S.A.). A 50 mm × 1.09 mm I.D. pre-column was connected to the main column inlet fitting and dry-packed in place with a pellicular C_{18} phase (Vydac Reverse Phase; Applied Science Labs., State College, Pa., U.S.A.) by vibration for 10 min. A 1/16-in. Swagelok union was attached and the constricted portion packed with glass wool. The entire system was then connected to a sample injection valve (Model CV-6-HPax, 50-μl sample loop; Valco Instruments, Houston, Texas, U.S.A.) with a short length of 1.5 mm × 0.25 mm I.D. tubing. A water jacket was also made for the pre-column from a length of 9.5 mm I.D. × 6.4 mm wall Tygon vacuum tubing. Water ports were made by boring holes in the wall and inserting short lengths of glass tubing. The ends were sealed at the 1/16-in. Swagelok nuts with O-rings. The entire column system was operated at 55°. The mobile phase was acetonitrile—water (25:75), flowing at 120 ml/h with a pressure of 10.3 MPa (1500 p.s.i.).

Procedure

To 0.50 ml of plasma in a 13 × 100 mm disposable culture tube are added, in order, 0.50 ml of internal standard, 1 ml of acetonitrile and 2 ml of iso-octane. The acetonitrile is added while vortexing to deproteinize the plasma. After addition of the iso-octane, the tube is vortexed for 15 sec to extract lipid material. The sample is centrifuged at 1000 *g* for 3 min, the iso-octane layer is aspirated along with the small amount of solid material at the interface, and the aqueous acetonitrile supernatant is decanted into a 10 × 75 mm disposable culture tube containing about 0.7 cm of granular potassium chloride. The tube is vortexed for 10 sec to saturate the solution and salt out the acetonitrile. After centrifugation for 5 min, the acetonitrile layer is transferred to a second 10 × 75 mm tube and evaporated to dryness at 50° under an air or nitrogen stream. The residue is dissolved in 140 μl of mobile phase, and 50 μl are injected into the liquid chromatograph. Quantitation is by peak-height ratio.

RESULTS AND DISCUSSION

Chromatograms produced by extracts of plasma samples drawn from epileptic patients are shown in Fig. 1. The sample that gave trace A was drawn from a patient taking phenytoin and methsuximide. Trace B was obtained with plasma drawn from a patient receiving carbamazepine and 1200 mg/day of I in four equal doses. The sample was taken 4 h after administration of 300 mg of I. Trace C was obtained with plasma drawn from a patient 1 h after administration of a final dose of I. A sample drawn two days later from this same patient gave trace D. This patient was also receiving phenobarbital, primidone and

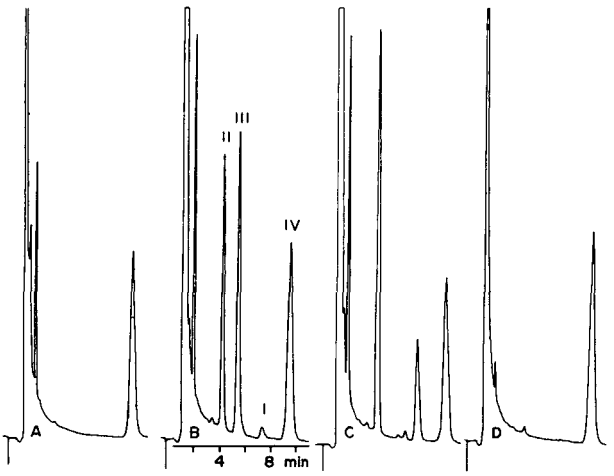


Fig. 1. Chromatograms of plasma extracts from epileptic patients. (A) Patient receiving phenytoin and methsuximide; (B) patient receiving carbamazepine (III) and cinromide (I), sample drawn 4 h after dose: (C) patient receiving phenobarbital, primidone, phenytoin and cinromide, sample drawn 1 h after final dose of cinromide; (D) same as C, sample drawn 2 days after final dose of cinromide. See text for drug concentrations. Peaks II and IV correspond to 3-bromocinnamamide and 3-bromo-N-isopropyl cinnamamide, respectively.

phenytoin. Concentrations of I and II, respectively, in traces B, C and D were 0.14 μg/ml and 1.99 μg/ml, 1.37 μg/ml and 4.39 μg/ml, and 0 μg/ml and 0.062 μg/ml, respectively. The carbamazepine concentration in trace B was 3.96 μg/ml.

The simple, clean extraction is based on the ability of acetonitrile to efficiently deproteinize plasma, its miscibility with dilute aqueous solutions and its immiscibility in potassium chloride-saturated solutions. The iso-octane wash eliminates lipid material that would be insoluble in the mobile phase but would be extractable into acetonitrile. The residue after evaporation of the acetonitrile is largely potassium chloride, which is soluble in the mobile phase and does not interfere with the analysis or contribute to degradation of the column. This procedure can also be used for efficient extraction of the barbiturate, hydantoin and succinimide anticonvulsants. Chromatographic conditions for quantitation of these compounds have been given by Adams and Vandemark [3]. Regeneration of the column system can be accomplished when necessary by successive elution with 100 ml each of acetonitrile, chloroform and acetonitrile, with injection of about 2 ml of dimethyl sulfoxide in small increments during the final acetonitrile wash.

Short-term reproducibility was assessed by extraction of six replicate samples containing 1.55 μg/ml, 2.01 μg/ml and 1.57 μg/ml of I, II and III, respectively. Relative standard deviations of peak-height ratios were 1.2%, 1.4% and 2.3%, respectively. Linearity of analytical curves and long-term reproducibility were checked by extraction of six plasma standards spiked with I, II and III to cover the concentration ranges of interest (up to 2.5 μg/ml I, 5.2 μg/ml II, and 11.1 μg/ml III). Correlation coefficients for all curves run over a 30-day period were greater than 0.999. Slopes of curves run on the 1st, 4th, 16th and 30th days had relative standard deviations of 2.8%, 3.3% and 2.9% for I, II and III, respectively.

Recoveries were assessed by comparison of extracted plasma standards with an analytical curve derived from nonextracted standards. For all samples, the internal standard was added to the final extract before evaporation. Recoveries of I, II and III were 61.7 ± 1.9%, 60 ± 5% and 61.0 ± 2.8% at concentrations of 1.0 μg/ml, 2.1 μg/ml and 4.5 μg/ml, respectively. By addition of standards at various stages of the extraction, losses at each step were estimated to be 13—15% during deproteinization; 7—11% during the iso-octane wash, aspiration and transfer; and 15—19% during the acetonitrile extraction. Recovery of all drugs can be increased by about 20% by using 0.25 ml of plasma and internal standard. When 0.5 ml of plasma and an internal standard concentration of 3 μg/ml are used, the lower limits of quantitation are 10—20 ng/ml for all drugs at a detector sensitivity of 0.016 a.u.f.s.

A dose—response relationship was determined for an epileptic female patient, weighing 51.8 kg, who was hospitalized during the study. All samples were drawn 4 h after the morning dose of I, which was given in 4 equal doses at 7 am, 12 noon, 5 pm, and 10 pm. A plot of plasma concentration (μg/ml) vs. dose (mg I per kg of body weight per day) was calculated from the average plasma levels obtained over a 3-day period, during which each dose was held constant. The correlation coefficients for I and II were 0.980 and 0.999,

respectively, and the slopes indicated plasma level changes of 0.00400 μg/ml I per mg I per kg per day and 0.0617 μg/ml II per mg I per kg per day. Preliminary pharmacokinetic data, obtained for four patients, indicated that peak concentrations of I and II were reached at 1 h and 2.5—3 h, respectively, after dosage. Half lives were about 1 h and 5—7 h for the parent drug and metabolite, respectively. More detailed clinical data are to be published in a future article.

The importance of heating the pre-column, which, in effect, only serves to pre-heat the mobile phase, is illustrated in Fig. 2. In each case a standard mix-

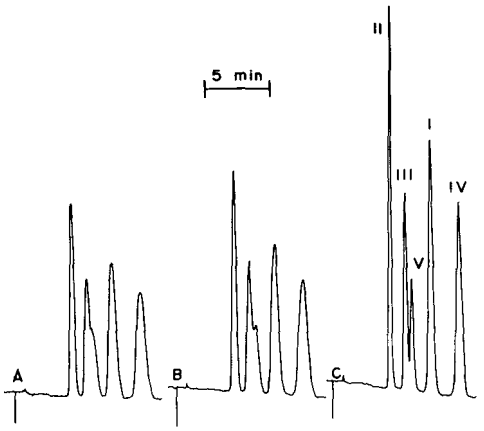


Fig. 2. Chromatograms of a standard mixture of I, II, III, IV and clonazepam (V) injected into (A) the main column at 55° with no pre-column; (B) the main column at 55° with pre-column at room temperature and (C) the main column at 55° with pre-column at 55°. Approximately 0.3 μg of each compound was injected in each case. Peak designations correspond to those of Fig. 1.

ture of the compounds used in this assay (I, II, III and IV) with the addition of clonazepam (a benzodiazepine anticonvulsant) was injected into the system. Mobile phase flow-rate was held constant. Trace A was obtained with the main column at 55° and no pre-column, trace B with the main column at 55° and the pre-colum at room temperature, and trace C with both the main column and pre-column at 55°. Clonazepam was added as a gauge of resolution because it was impossible to separate this compound from carbamazepine on the main column alone in a reasonable time with acetonitrile—water or methanol—water mobile phases. The number of theoretical plates was calcuated for each system based on the final peak (IV) from the equation: $N = 16\ (X/Y)^2$, where N is the number of the theoretical plates, X is the retention time and Y is the base width of the peak of interest. Respective efficiencies for systems A, B and C were 831, 973 and 2588 theoretical plates.

This effect does not represent a real increase in efficiency of the analytical column because if the system were run at room temperature, the higher plate count would be observed. The peak broadening in chromatograms A and B is not a characteristic of the column, but is a result of the thermal nonequilibrium that is produced when cold mobile phase enters a heated column. The analyst who does reversed-phase chromatography at increased temperatures without

pre-heating his mobile phase may be working with a serious handicap. A more detailed discussion of behavior of reversed-phase columns operated at increased temperatures is in press [4].

Preliminary data on another metabolite of cinromide, 3-bromocinnamic acid (VI), shows that it may also have some anticonvulsant effect. This compound can be analyzed by this method with only a slight change in mobile phase composition. By buffering the mobile phase to pH 4.7 with NaH_2PO_4 (0.015 mole/l) and adding tetraethylammonium perchlorate (0.005 mole/l), an ion-pairing effect is achieved that increases the retention time of the acid. Fig. 3

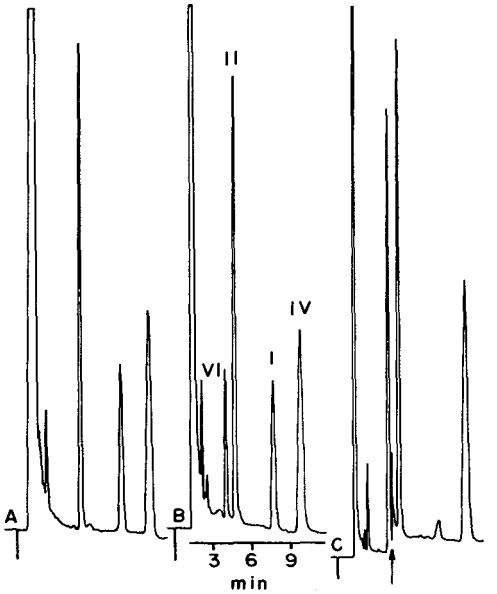


Fig. 3. Chromatograms of an extract of a plasma sample containing 1.86 μg cinromide (I) per ml, 3.50 μg 3-bromocinnamamide (II) per ml and 1.53 μg 3-bromocinnamic acid (VI) per ml run with (A) acetonitrile—water (25:75), and (B) acetonitrile—water (25:75) with NaH_2PO_4 (0.015 mole/l, pH 4.7) and tetraethylammonium perchlorate (0.005 mole/l) added. (C) Chromatogram of extract of plasma drawn from a patient taking cinromide. Arrow indicates change in detector sensitivity from 0.512 to 0.064 a.u.f.s. Concentrations are 0.21 μg/ml I, 3.01 μg/ml II and 28.4 μg/ml VI.

shows chromatograms of an extract of a plasma standard containing the parent drug and the two metabolites run with the normal mobile phase (A) and the ion-pairing mobile phase (B). In chromatogram A the acid metabolite elutes with the solvent front. Chromatogram C shows an extract of plasma drawn from a patient taking cinromide. The arrow indicates a change in sensitivity of the detector from 0.512 to 0.064 a.u.f.s. to accommodate the high concentration of acid (up to 30 μg/ml). Linearity has been confirmed up to 33 μg/ml VI with quantitation by peak-height ratio. No adjustment of peak heights is necessary as long as the ratio of the sensitivity setting for the acid to that for the internal standard is kept constant.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Service of the Veterans Administration, the Epilepsy Research Foundation of Florida, Inc., Clinical Research Center Grant RR-82 and Burroughs-Wellcome Co. Dr. Bruni is supported by the Ministry of Health of Ontario, Canada.

The authors thank Mitchell Thomas and Dora Mitchell for technical assistance and Barbara Barbour and Marilyn Paul for clinical assistance. Thanks are also due to Richard M. Welch and Shirley Hsu of Burroughs-Wellcome Co. for their generous gifts of the compounds used in this study and helpful discussions.

REFERENCES

1 Burroughs-Wellcome, Research Report on the Pharmacology and Toxicology of B.W. 122 (Cinromide), 1975.
2 R.J. Perchalski and B.J. Wilder, J. Chromatogr., 145 (1978) 97.
3 R.F. Adams and F.L. Vandemark, Clin. Chem., 22 (1976) 25.
4 R.J. Perchalski and B.J. Wilder, Anal. Chem., 51 (1979) in press.

Journal of Chromatography, 163 (1979) 195—200
Biomedical Applications

CHROMBIO. 331

QUANTITATIVE THIN-LAYER CHROMATOGRAPHIC MEASUREMENT OF N-TRIFLUOROACETYLADRIAMYCIN-14-VALERATE (AD 32) AND TRIFLUOROACETYLADRIAMYCIN (AD 41) IN BLOOD AND TISSUES

B. BARBIERI, R. ABBRUZZI, A. BENIGNI, M. RIZZARDINI, M.G. DONELLI, S. GARATTINI and M. SALMONA*

Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan (Italy)

(Received December 28th, 1978)

SUMMARY

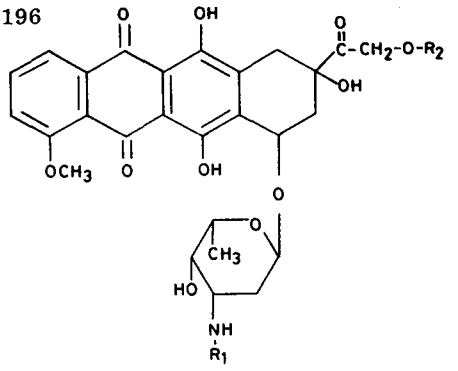
A thin-layer chromatographic method has been developed for the detection and measurement of N-trifluoroacetyladriamycin-14-valerate (AD 32) and its major metabolite trifluoroacetyladriamycin (AD 41). The procedure gives satisfactory linearity over a large range of concentrations. The coefficient of variability is about 10% over the entire range of usable concentrations, giving good reproducibility; sensitivity is 25 ng for both AD 32 and AD 41. Analysis is specific for AD 32 and AD 41 since adriamycin or more polar metabolites can be differentiated. Recovery is high (85—90%) and the method is simple and economical to use. Pharmacokinetics of AD 32 and AD 41 are reported in blood and some tissues of mice bearing Lewis Lung carcinoma.

INTRODUCTION

N-Trifluoroacetyladriamycin-14-valerate (AD 32) is a recent derivative of adriamycin [1—3] reported to display greater antitumoral activity in animals than the parent compound. Recently it was introduced in a (clinical trial) phase I study. The chemicophysical and biological characteristics of this compound are completely different from those of adriamycin, and its mechanism of action is thus still unclear. For instance, it is completely insoluble in water, does not intercalate with DNA [4, 5] and the mode of its cytotoxic action [6, 7] appears to differ, at least in part, from that of adriamycin.

The ester bond at position 14 formed by adriamycin and valeric acid is easily split by blood and tissue esterases, giving rise to the metabolite N-trifluoroacetyladriamycin (AD 41), (Fig. 1), whereas the amide bond between the amino group on the glycosidic moiety and trifluoroacetic acid seems relatively stable [8]. AD 41 has been reported to maintain the inability of AD 32 to in-

*To whom correspondence should be addressed.



	R_1	R_2
Adriamycin	H	H
AD 32	$COCF_3$	COC_4H_9
AD 41	$COCF_3$	H

Fig. 1. Structures of adriamycin, N-trifluoroacetyl-adriamycin-14-valerate (AD 32) and N-trifluoroacetyl-adriamycin (AD 41).

tercalate with DNA but it is cytotoxic [5] and shows antitumoral activity in vivo [9].

The metabolism of AD 32 was studied in detail by Israel and co-workers [10, 11]. They found AD 41 in blood, urine and bile together with more polar metabolites such as 13-dihydro-N-trifluoroacetyladriamycin-14-valerate (AD 60) and N-trifluoroacetyladriamycinol (AD 92) and traces of adriamycin and adriamycinone. AD 41 was always the major metabolite. So far no data are available on the tissue distribution of AD 32 and its metabolites.

In this paper we present a simple method of measuring levels of AD 32 and AD 41 routinely in blood and tissues. This method is less senstive than that presented by Israel et al. [11], based on thin-layer chromatographic (TLC) separation followed by high-pressure liquid chromatographic analysis, but is less time-consuming and permits a large number of analyses to made easily, which is an important prerequisite for accurate pharmacokinetic studies. Furthermore, since a sufficiently specific assay of adriamycin and its derivatives in tissues is always a serious analytical problem, the method described in this paper may be of interest for the study of the pharmacological effects of AD 32 and its metabolites.

MATERIALS AND METHODS

Chemicals

All solvents were reagent grade (Carlo Erba, Milan, Italy). Silica gel plates 20 × 20 cm, 0.25 mm thick, without fluorescence indicator and without calcium sulphate binder were purchased from Merck, Darmstadt, G.F.R. N-Trifluoroacetyladriamycin-14-valerate (AD 32), N-trifluoroacetyladriamycin (AD 41), adriamycin, adriamycinol and daunomycinone were kindly supplied by Farmitalia, Milan, Italy. AD 32 and AD 41 were dissolved at a concentration of 6 mg/ml in saline containing 10% Tween 80. N-Trifluoroacetyladriamycinol (AD 92) was a kind gift of Dr. Mervyn Israel, Sidney Farber Medical School, Boston, Mass., U.S.A.

TLC scanning

Fluorescence scans were made with a Turner 111 TLC scanner, at an excitation wavelength of 475 nm and emission at 580 nm, connected with a Kontron W + W 1001 recorder.

Animals

C57B1/6 male mice (20 ± 2 g body weight), obtained from Charles River Italy (Calco, Italy), were used for these experiments. The animals received an intramuscular transplant of 2×10^5 viable cells of the Lewis Lung carcinoma, maintained by passages in the same strain every two weeks.

AD 32 was administered intravenously at a dose of 80 mg/kg 14 days after tumor transplantation. Animals were killed at various intervals of time after drug administration, and blood, heart, tumor and spleen were immediately collected. Tissues were repeatedly washed in chilled saline to eliminate blood contamination. Four animals were used each time.

Extraction procedure

Blood was collected in heparinized tubes and immediately cooled to 0°; 0.5 ml of blood was then mixed with 0.8 ml chilled water and deproteinized by the addition of 0.2 ml of cold acetone. These operations normally took less than 2 min. Table I gives a comparison of AD 32 hydrolysis at 0°, 25° and 37° by blood esterases. Within the first 2 min at 0° and 25° less than 10% of the drug is hydrolysed, whereas at 37° during the same period 45% of the compound is hydrolysed. The samples were extracted twice with 3 ml of diethyl ether, and the ethereal phases pooled and taken to dryness under a gentle stream of nitrogen. Frozen tissues were homogenized with an Ultra Turrax apparatus, Model TP 18/2N (Jane and Kungel, Staufen, G.F.R.) in chilled water in ratios of 1:4 or 1:10 (g/ml), and 0.2 ml of cold acetone was added to 0.5—1.0 ml samples. The samples were extracted twice with 3 ml diethyl ether and taken to dryness as described above.

TABLE I

HYDROLYSIS OF AD 32 BY BLOOD

Ten micrograms of AD 32 were added to 1 ml of control C57B1/6 mice blood. The mixture was incubated at different temperatures for 2—30 min and AD 32 and AD 41 were measured as described in the text. Each value is the mean of three determinations.

Incubation time (min)	Hydrolysis (%)		
	0°	25°	37°
2	8.0	6.0	45
5	8.9	13.0	49
10	9.7	38.0	48
30	10.0	72.0	62

Sample spotting

The residues were dissolved in 100—200 μl of an acetone solution of daunomycinone (20 μg/ml) used as standard; 5 μl were spotted on a silica gel plate

using a micropipette (Camag). On each plate eight unknown samples were spotted with four internal standards obtained by adding 0.05—2 μg of AD 32 and AD 41 to blood or tissue from control animals, and extracting as described for unknown samples. The plates were developed to 15 cm from the origin in a lined tank and were then removed and dried briefly before scanning.

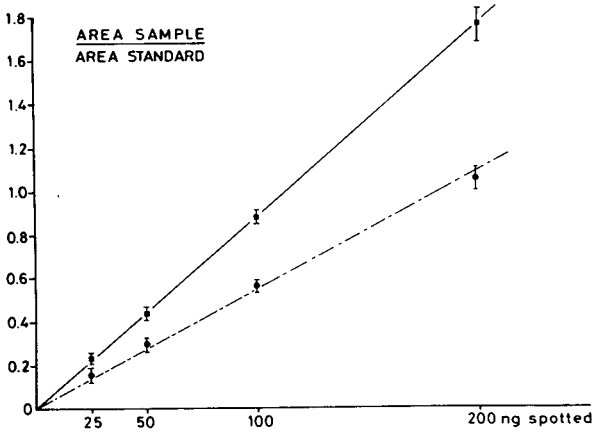
RESULTS AND DISCUSSION

Linearity and reproducibility

As shown in Fig. 2 the standard curve for this method was linear in the range 25—200 ng for AD 32 and AD 41. Each value is the mean ± S.E. of 15 different determinations. The reproducibility of the method was determined by scanning five times three different plates on which 25—200 ng AD 32 and AD 41 were spotted together with a known amount (100 ng) of daunomycinone as internal standard. The coefficient of variation for this analysis is 11.5 ± 2.2% for AD 32 and 9.3 ± 2.1% for AD 41.

Recovery

Recovery of AD 32 and AD 41 from blood and tissues was established by comparing the TLC peak areas for samples with AD 32 or AD 41 added with those on plates where the same amount of AD 32 and AD 41 had been spotted directly. Recovery was 90 ± 5% for blood and 85 ± 7% from tissues.



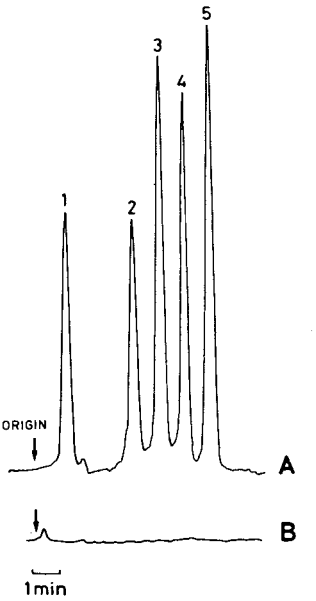


Fig. 2. Standard curves for AD 32 and AD 41. Mean and S.E. of 15 determinations are given at each concentration. ▪, AD 32; •, AD 41.

Fig. 3. (A) TLC scanning of 50 ng AD 92 (peak 1), 50 ng AD 41 (peak 2), 100 ng adriamycinone (peak 3), 75 ng AD 32 (peak 4) and 100 ng daunomycinone (peak 5) added as internal standard. (B) TLC scanning of a control blood sample.

Resolution and specificity

Development of the plates with the solvent system chloroform—methanol—acetic acid (93:5:2) gave the following R_F values: daunomycinone, 0.79; AD 32, 0.64; adriamycinone, 0.62; AD 41, 0.43; AD 92, 0.14 (Fig. 3A). Under the conditions employed adriamycin and adriamycinol do not move. Moreover, these two compounds are not recovered at all from blood and tissues. Thus the only possible interfering metabolites are the non-polar ones. Blood and tissues blanks did not give any interfering peaks during the TLC analysis (Fig. 3B).

Pharmacokinetics of AD 32

Fig. 4 shows the kinetics of AD 32 and AD 41 in blood after a single intravenous injection of AD 32 (80 mg/kg) in C57Bl/6 mice bearing Lewis Lung carcinoma. AD 41 is detectable almost immediately after injection, suggesting that biotransformation of AD 32 into its major metabolite is very rapid. Their rates of disappearance from blood are quite different, AD 32 having a K_{el} of 0.20 (μg/ml), almost three times that of AD 41, suggesting that the metabolite is present in the blood compartment in higher amounts and for longer than its parent compound. Table II reports the peak levels of AD 32 and AD 41, the corresponding areas under the curve and half-lives ($T_{½}$) in blood and in the tumor, heart and spleen. Except in the blood, the AD 41 peak levels are always higher than AD 32 peaks; the areas under the curves 24 h after treatment and the half-lives indicate clearly that tumor, heart and spleen are much more exposed to AD 41 than to AD 32. The significance of these data deserves more studies, in particular to establish (a) if AD 32 is active per se or if it acts as a pro-drug, being hydrolysed by blood and tissue esterases, and (b) if the tissue distribution of AD 41 is simply dependent on the presence of esterases or depends also on its chemicophysical characteristics.

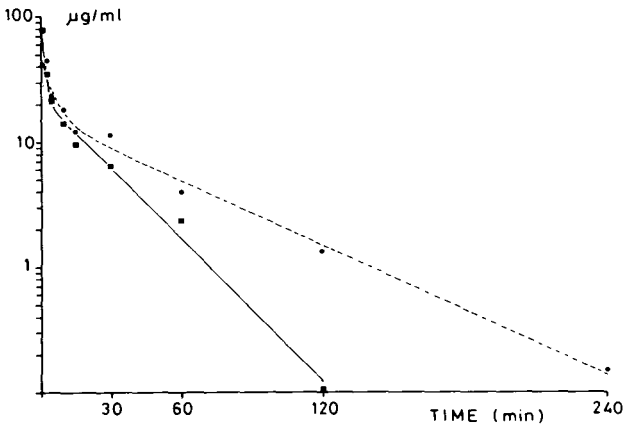


Fig. 4. Blood levels of AD 32 and AD 41 after intravenous injection of AD 32 (80 mg/kg) in C57Bl/6 mice bearing Lewis Lung carcinoma. ▪———▪, AD 32; ●- - - -●, AD 41.

TABLE II

BLOOD AND TISSUE LEVELS OF AD 32 AND AD 41 AFTER INTRAVENOUS ADMINISTRATION OF AD 32 (80 mg/kg) TO C57B1/6 MICE BEARING LEWIS LUNG CARCINOMA

Treatment was given on day 14 after tumor transplantation. In parentheses the time of peak concentration. The area under the concentration versus time curve (AUC) was measured by trapezoidal integration up to the last time at which the drug was detectable. The half-life ($T_{½}$) was assessed by calculating the significance of the regression using the determination coefficient r^2 (least-squares method).

	Peak level (μg/ml) or (g)		AUC after 24 h (μg/ml) or (g × min)		Half-life (min) or (h)	
	AD 32	AD 41	AD 32	AD 41	AD 32	AD 41
Blood	77.1 ± 2.4 (1 min)	44.8 ± 0.5 (3 min)	705 ± 65	990 ± 187	16 min	35 min
Tumor	5.2 ± 0.6 (10 min)	19.3 ± 1.5 (30 min)	161 ± 15	11,189 ± 1210	21 min	16 h
Heart	7.4 ± 2.6 (1 min)	87.0 ± 4.4 (1 min)	569 ± 55	7,002 ± 977	8 min	1.4 h
Spleen	11.8 ± 1.4 (1 min)	108 ± 5.7 (30 min)	72 ± 19	20,075 ± 1684	N.D.*	1.15 h

*N.D. = Pharmacokinetic analysis not possible because of insufficient data.

ACKNOWLEDGEMENT

This work was supported by NCI, Contract No. NO1-CM6-7064.

REFERENCES

1 M. Israel, E.J. Modest and E. Frei III, Cancer Res., 35 (1975) 1365.
2 M. Israel, S.K. Tinter, H. Lazarus, B. Brown and E.J. Modest, in International Union Against Cancer, International Cancer Congress, Florence, Italy, October 25, 1974, Casa Editrice Ambrosiana, Milan, 1974, Abstracts V-4, p. 752.
3 A. Krishan, M. Israel, E.J. Modest and E. Frei III, Cancer Res., 36 (1976) 2108.
4 T. Facchinetti, A. Mantovani, L. Cantoni, R. Cantoni and M. Salmona, Chem.-Biol. Interactions, 20 (1978) 97.
5 T. Facchinetti, A. Mantovani, R. Cantoni, L. Cantoni, C. Pantarotto and M. Salmona, Biochem. Pharmacol., 26 (1977) 1953.
6 A. Vecchi, M. Cairo, A. Mantovani, M. Sironi and F. Spreafico, paper presented at the 10th International Congress of Chemotherapy, Zurich, September 18—23, 1977, in press.
7 A. Vecchi, M. Cairo, A. Mantovani, M. Sironi and F. Spreafico, Cancer Treat. Rep., 62 (1978) 111.
8 M. Israel, P.M. Wilkinson, W.J. Pegg and E. Frei III, Cancer Res., 38 (1978) 365.
9 M. Israel, W.J. Pegg, R. Seshadri and L.M. Parker, Proceedings of the 5th International Symposium on Medicinal Chemistry, Paris, July 1976, p. 63.
10 L.M. Parker, M. Israel, M. Hirat, E. Modest and E. Frei III, Proc. Amer. Ass. Cancer Res., 17 (1976) 108.
11 M. Israel, W.J. Pegg and P.M. Wilkinson, J. Pharmacol. Exp. Ther., 204 (1978) 696.

Journal of Chromatography, 163 (1979) 201—205
Biomedical Applications

CHROMBIO. 330

Note

Simple gas chromatographic screening procedure for lactic and pyruvic acids in human plasma

DAVID V. McCALLEY and MICHAEL COOKE*

Department of Chemistry, The University of Bristol, Cantocks Close, Bristol BS8 1TS (Great Britain)

and

CHARLES A. PENNOCK

Department of Child Health, The University of Bristol, St. Michaels Hill, Bristol BS2 8BJ (Great Britain)

(First received November 17th, 1978; revised manuscript received February 20th, 1979)

The presence of increased amounts of various organic acids in human blood plasma has been identified in a variety of diseases. Although some of these are rare and hereditary there are a number of more widespread conditions in which this occurs. One of these is lactic acidosis, a condition which results in the presence of increased amounts of lactic acid in plasma and urine, generally accompanied by elevated pyruvic acid levels.

The determination of organic acids in biological fluids is a difficult analytical problem because of the polar and hydrophilic nature of these compounds. The use of solvents such as diethyl ether and ethyl acetate for the extraction of acidic metabolites from acidified plasma or urine is not only non-quantitative because of the unfavourable partition coefficients for these acids between the solvents and water, but also results in the extraction of non-acidic components [1]. Steam distillation has been used [2—4] to extract organic acids but often causes an intolerable dilution of the relevant compounds.

The determination of organic acids by gas chromatography usually requires a suitable derivatization procedure because of the polar nature and low thermal stability of the parent compounds [5, 6]. However, some reports of the direct determination of organic acids on conventional gas—liquid chromatographic

*To whom correspondence should be addressed.

columns have appeared [7, 8]. Pyruvic and lactic acids may be assayed by an enzymatic method

$$\text{pyruvate} \xrightleftharpoons{\text{(lactate dehydrogenase)}} \text{lactate}$$
$$\text{NADH} + \text{H}^+ \longleftrightarrow \text{NAD}$$

but the procedure is rather complex as each acid has to be determined separately. Paper and thin-layer chromatographic methods have also been used to detect the presence of organic acids in biological fluids but these procedures are only semi-quantitative.

In this preliminary report we describe a simple gas chromatographic assay for lactic and pyruvic acids in human plasma. Plasma, rather than urine, was chosen because it contains fewer constituents than urine, and because lactic acid is present in negligible amounts in the urine of normal subjects.

MATERIALS AND METHODS

Chromatography was carried out on a Pye-Unicam GC-V gas chromatograph equipped with dual flame ionisation detectors, and a 90 cm × 0.3 cm coiled glass column packed with Tenax G.C. (35—60 mesh) (Applied Science Labs., State College, Pa., U.S.A.) conditioned according to the manufacturer's recommendations (Applied Science Laboratories Technical Bulletin No. 24). A nitrogen carrier gas flow-rate of 15 ml min^{-1} and a column temperature of 155° were employed. Standard acids were obtained from BDH (Poole, Great Britain).

Blood samples were obtained 10—14 h after the last meal. Deproteinisation was accomplished by adding blood to an equal volume of 5% perchloric acid. After standing for 15 min the sample was centrifuged at 2600 *g* for 20 min. Excess perchloric acid was removed prior to analysis by neutralisation with a buffer solution containing 20% (w/v) potassium hydroxide and 1 *M* in triethanolamine. Re-acidification with concentrated HCl to give an approximately 0.1 *M* acid solution was followed by injection onto the gas chromatographic column.

Gas chromatography—mass spectrometry was performed on a V.G. Micromass 16B2 mass spectrometer interfaced to a Pye 104 gas chromatograph via a single-stage jet separator. Helium was used as the carrier gas.

RESULTS AND DISCUSSION

Using these conditions the retention times of pyruvic and lactic acids were 2.6 and 6.5 min, respectively. For batch analyses the clear supernatant after centrifugation may be decanted and stored until required.

Standard solutions of lactic and pyruvic acids corresponding to, and above, the normal values found in human plasma gave a linear response for the required ranges of 0—50 $\mu g\ ml^{-1}$ (regression equation $y = 4.379x + 5.69$, correlation coefficient 0.9926) for pyruvic acid, and 0—350 $\mu g\ ml^{-1}$ (regression equation $y = 2.212x - 25.27$, correlation coefficient 0.9971) for lactic acid. The levels in the plasma of the subjects studied ranged over 90—160 $\mu g\ ml^{-1}$ for lactic acid and 3.5— 7.0 $\mu g\ ml^{-1}$ for pyruvic acid.

As no suitable cases of lactic acidosis were available for study, blood samples from subjects undergoing fructose loading tests were analysed. It has been reported [9] that about 35% of the fructose load is converted to lactate and pyruvate, the remainder being converted to glucose. Subjects were in the fasting state prior to the first sampling. Subsequent to the first sampling 1 g of fructose per kg of body weight was administered. Further blood samples were taken after 30 min and 45 min. Gas chromatographic analysis (duplicate injection) was performed after the sample preparation procedure described above. Typical results obtained for one of the subjects studied are displayed in Table I, and Fig. 1b—d. Fig. 1a is a chromatogram of standard pyruvic, lactic and hydroxybutyric acids.

TABLE I

LACTIC AND PYRUVIC ACID LEVELS IN A FRUCTOSE-LOADED SUBJECT

Sample No.	Time (min)	Pyruvic acid (μg/ml)	Lactic acid (μg/ml)
1	0	6.0	120
2	30	8.2	290
3	45	8.2	330

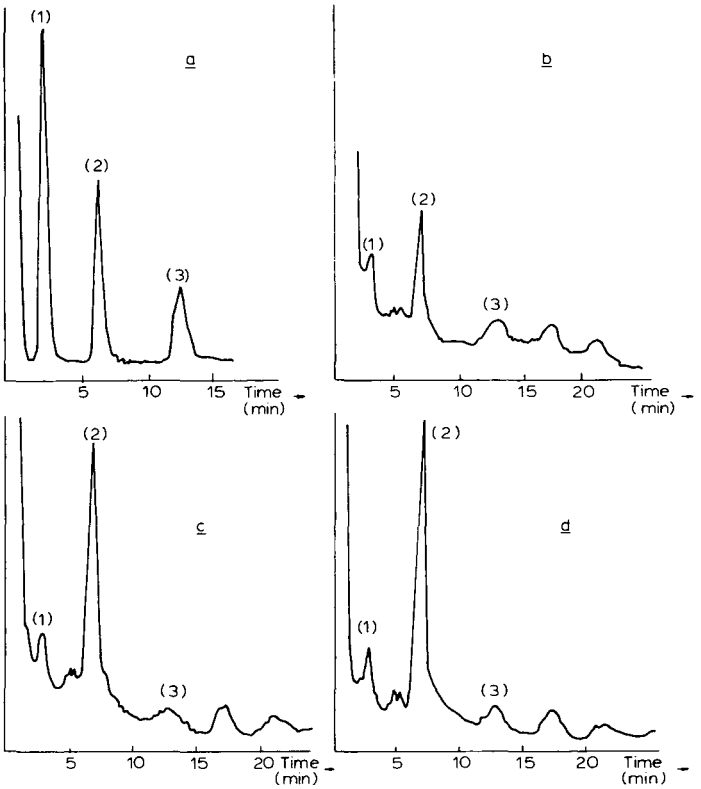


Fig. 1. Chromatograms of (a) pyruvic (1) lactic (2) and hydroxybutyric (3) acid standards; (b) normal plasma; (c) plasma 30 min after fructose loading; (d) plasma 45 min after fructose loading.

It was apparent from the chromatograms obtained that there were other constituents present in the sample, but no interference by any biologically significant organic acids from any major metabolic pathway has been observed. Co-injection with standards suggested that one of these constituents was hydroxybutyric acid (t_R = 12.8 min). The co-determination of hydroxybutyric acid is particularly desirable since there is a close relationship between hydroxybutyric acid levels and the occurrence of lactic acidosis [10, 11]. Confirmation of column eluates by gas chromatography—mass spectrometry proved unsuccessful. Presumably the underivatized acids do not survive transfer from the column to the mass spectrometer source via an interface maintained at 220°.

After injection of about ten samples of treated plasma solution the top 1—2 cm of the column became visibly contaminated by the build-up of non-volatile material from the injected sample. Although this was observed not to affect column performance the top of the column was renewed periodically (approximately every 25 injections). Frequent injections of solvent (0.1 *M* HCl) were carried out to preserve column performance. Ghosting (i.e. the observation of spurious peaks) was caused by the presence of a glass-wool plug (silanized or unsilanized) at the front end of the column. Glass wool should not be used to contain the column packing for this assay.

Quantitation for this work was accomplished by the measurement of peak areas. However, use of an internal standard should provide improved accuracy. Pivalic acid has been recommended [8] for this purpose but was found to be unsuitable as it eluted as a broad band at the temperature employed for the analysis. Its hygroscopicity and unpleasant smell are further disadvantages. *n*-Butanoic, *n*-pentanoic and *n*-hexanoic acids were also evaluated. Of these three *n*-hexanoic has an ideal retention time, being eluted after lactic acid and before hydroxybutyric acid. Unfortunately the low solubility of *n*-hexanoic acid in an aqueous medium precludes its use as an internal standard.

CONCLUSIONS

The simultaneous determination of pyruvic, lactic and possibly of hydroxybutyric acids at the levels found in human plasma may be achieved using the porous packing Tenax G.C. Small sample volumes (ca. 250 μl) are needed, little sample preparation is necessary and automation to cope with routine analysis should be possible. The development of a suitable internal standard would be an added advantage, although, due to the gross nature of many metabolic disorders, it can be argued that the proposed method using peak areas and calibration curves is sufficiently accurate as it stands.

REFERENCES

1 E. Jellum, J. Chromatogr., 143 (1977) 427.
2 K. Tanako, M.A. Budd and M.L. Efron, Proc. Nat. Acad. Sci. U.S., 56 (1966) 236.
3 F.A. Hommes, J. Kuipens, J.O. Elma and J.F. Tansen, Pediat. Res., 2 (1968) 519.
4 D. Gompertz and G.D. Draffan, Clin. Chim. Acta, 40 (1972) 5.
5 R.A. Chalmers and A.M. Lawson, Chem. Brit., 8 (1975) 290.
6 R.A. Chalmers and R.W.E. Watts, Analyst (London), 97 (1972) 224.

7 H. von den Berg and F.A. Hommes, Clin. Chim. Acta, 51 (1974) 225.
8 R.A. Chalmers, A.M. Lawson and R.W.E. Watts, Clin. Chim. Acta, 52 (1974) 31.
9 H.F. Woods, L.V. Eggleston and H.A. Krebs, Biochem. J., 119 (1972) 501.
10 J.E. Pettersen, S. Landaas and L. Eldjarn, Clin. Chim. Acta, 48 (1973) 213.
11 S. Landaas and J.E. Pettersen, Scand. J.Clin. Lab. Invest., 35 (1975) 259.

Journal of Chromatography, 163 (1979) 206—211
Biomedical Applications

CHROMBIO. 324

Note

Improvements in the gas chromatographic analysis of acetylcholine and choline

J.W. KOSH, MARTHA B. SMITH, J.W. SOWELL and J.J. FREEMAN

Division of Pharmacology, College of Pharmacy, University of South Carolina, Columbia, S.C. 29208 (U.S.A.)

(Received November 8th, 1978)

Since the mid-sixties, there have been several reports of chemical methods for the determination of choline and acetylcholine. The chemical methods have the advantages of specificity, selectivity, reliability and in some cases sensitivity over bioassay methods. An excellent review of the chemical methods in current use has been compiled by Hanin [1].

Several reports in the literature of spurious rat brain choline and acetylcholine values were apparently due not only to the method of determination but to the post-mortem activity of several enzymes in the brain [2]. The use of high-power (5 kW) microwave irradiation has stabilized the values obtained for physiological levels of choline and acetylcholine [3].

In general, chemical methods can be divided into three groups: isotope derivative, fluorescence, and chromatographic methods. Gas chromatographic methods for cholinergic compounds differ primarily in their means of detection — mass spectrometry [4], nitrogen [5] or flame ionization [6] — and by their means of volatilizing acetylcholine and choline — demethylation [7] or pyrolysis [8].

This note describes advances in the gas chromatographic analysis of acetylcholine and choline and the incorporation of these techniques into previously reported methods [9].

EXPERIMENTAL

Materials

2,2',4,4',6,6'-Hexanitrodiphenylamine (dipicrylamine), butyryl chloride and 4-dimethylamino-3-methyl-2-butanone were obtained from Aldrich, Milwaukee, Wisc., U.S.A. Triton X-100 and 10% OV-17 on Gas-Chrom Q were obtained from Applied Science Labs., Richmond, Calif., U.S.A. Tris(hydroxymethyl methylaminopropane sulphonic acid) (TAPS) was obtained from Calbiochem, San Diego, Calif., U.S.A.

Synthesis of butyrylcholine iodide

Butyryl chloride (0.15 moles, 16.1 g) was added to 100 ml of acetone in a 500-ml round-bottomed flask in an ice-bath. 2-(N,N-Dimethylamino)ethanol (0.14 moles, 12.5 g) was dissolved in an equal volume of acetone and added slowly with stirring. The solution was stirred overnight under anhydrous conditions, cooled in an ice—acetone mixture and filtered in a büchner funnel. The white precipitate was washed two times with cold acetone and dried under vacuum.

The yield of the N,N-dimethylamino ethylbutyrate formed was 65%. Anhydrous diethyl ether (100 ml) was then added to the compound in a separatory funnel. Sodium hydroxide (0.91 moles, 3.6 g) was dissolved in 50 ml of water and then added to the solution. The water layer was then washed three times with 25 ml of ether. The ether extracts were combined and washed two times with 50 ml salt-saturated water. The ether layer was then filtered through a funnel containing anhydrous sodium sulfate. Methyl iodide (0.10 moles) was then added to the ether solution. Yellow crystals were precipitated from the solution after storing it in the freezer for 3½ h. The crystals were isolated, washed with acetone and tested for contamination by choline, which proved to be less than 1%.

Preparation of the column

OV-17 (10% w/w) on Gas-Chrom Q was pre-sifted through an 80-mesh sieve. Three grams of the 10% OV-17 were added to 10 ml acetone in a 100-ml round-bottomed flask. Triton X-100 (300 mg) was dissolved in 10 ml methanol and added to the flask. The suspension was blown dry with a nitrogen stream while rotating the flask by hand. The column packing was then dried in an oven for 12 h at 100°. The material was then put in a silanized glass column (1.2 m × 2 mm I.D.) and conditioned for 24 h at 150°.

A Beckman GC-65 dual-column gas chromatograph equipped with flame ionization detector was used. The gas flow-rates were: helium 40 ml/min, air 250 ml/min, and hydrogen 50 ml/min. The conditions were: column temperature 115°, injection port 200°, detector 225°.

Analysis of acetylcholine and choline

A gas chromatographic—mass spectrometric procedure for the micro-estimation of acetylcholine and choline [4, 9] was modified to allow for the gas chromatographic assay of acetylcholine and choline using butyrylcholine as an internal standard. Utilization of dipicrylamine to extract acetylcholine and choline into dichloromethane was retained. A new column material utilizing readily available materials (10% Triton X-100 and 10% OV-17 on Gas-Chrom Q) gave good results.

Following dissection of the brain from the skull, the brain was weighed and 50 nmoles of butyrylcholine were added to 4 ml acetone—1 *N* formic acid (85:15) for homogenization and analysis of whole brain. For half a brain or less, 2 ml acetone—1 *N* formic acid (85:15) were used. The homogenate was centrifuged at 26,000 *g* for 20 min. Two milliliters or the entire supernatant were transferred to a 10-ml screw-capped centrifuge tube. Two volumes (i.e. 4 ml) of diethyl ether were added to the tube, vortexed and centrifuged for

2 min. The ether phase was aspirated and residual ether and acetone distilled under a stream of nitrogen in a water-bath at 80° for 5 min. 2 *M* TAPS buffer (0.5 ml) (pH 9.2) was added to 2 ml of 2 m*M* dipicrylamine in dichloromethane and vortexed for 2 min followed by 2 min centrifugation. The upper aqueous layer was discarded and the organic phase transferred to another tube. The dichloromethane was evaporated in a stream of dry nitrogen. The sample was vacuum desiccated for 5 min. To the dried sample was added 0.5 ml 5 m*M* silver *p*-toluenesulphonate in acetonitrile and 50 μl double-distilled propionyl chloride. After mixing, the sample was left at room temperature for 5 min, and the solution evaporated with a stream of dry nitrogen; 0.5 ml of 50 m*M* sodium benzenethiolate and 25 m*M* benzenethiol in butanone was then added to the residue. The air in the tube was displaced by dry nitrogen, and the sealed tube was incubated at 80° for 30 min. The cooled sample was treated with 100 μl 0.5 *M* citric acid and washed three times with 1 ml pentane. Traces of pentane were evaporated with a stream of dry nitrogen. The tube was put in an ice-bath and 25 μl chloroform were added. Then 2 *M* ammonium citrate—7.5 *M* ammonium hydroxide buffer (0.1 ml) was added and the sample mixed for 2 min on a vortex mixer, and centrifuged for 2 min on a clinical centrifuge. A 2-μl aliquot of the chloroform extract was injected into the gas chromatograph for analysis. Reference data for calibration of the assay were obtained as a part of each experiment using 50 nmoles butyrylcholine as internal standard. Peak height ratios were obtained by dividing the acetylcholine and choline peak heights by the butyrylcholine peak height. The recovery of acetylcholine, choline and butyrylcholine was approximately 80% through the method when compared to the direct injection of 50 nmoles of 4-dimethylamino-3-methyl-2-butanone.

RESULTS AND DISCUSSION

Fig. 1 demonstrates the linearity of the method. The insert demonstrates that the method is linear below 10 nmoles. The sensitivity of the method is acetylcholine 0.5 ± 0.01 nmoles (S.E.), choline 0.5 ± 0.03 nmoles (S.E.). Butyrylcholine (50 nmoles) is used as internal standard in all of the tubes. A 50-nmole equimolar mixture of acetylcholine, choline and butyrylcholine gives a peak height ratio of approximately one. The linearity of choline is lost at concentrations above 400 nmoles. This is due to a loss of extraction efficiency above 400 nmoles and is correctable by a double extraction with 2 m*M* dipicrylamine. When the extraction volumes are combined, taken to dryness and the assay continued, the curve then remains linear above 400 nmoles.

Fig. 2A illustrates a tracing of an equimolar mixture (50 nmoles of acetylcholine, choline and butyrylcholine) after injecting 2 μl of chloroform into the gas chromatograph. The equimolar mixture was started at the beginning of the assay in a formic acid—acetone mixture. The peaks are symmetrical and narrow enough to allow for measurement of peak heights as well as area determination if an integrator is available. The efficiency of the column, expressed in theoretical plates per foot, is 113 for acetylcholine, 204 for choline, and 238 for butyrylcholine. The column has been in use six months and has not shown signs of deterioration.

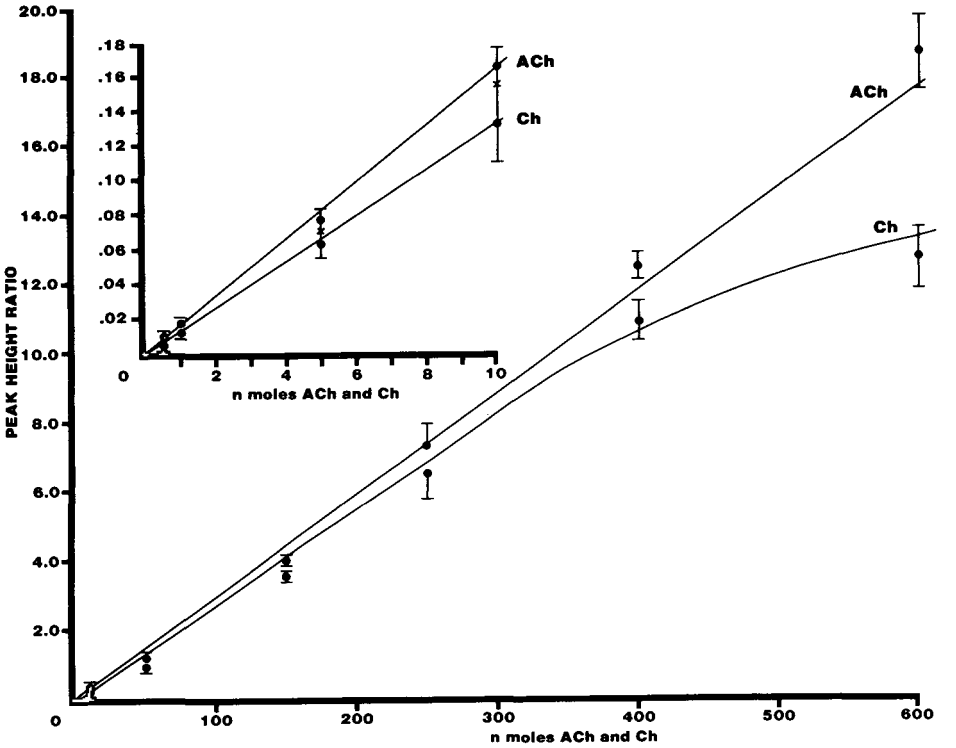


Fig. 1. Peak height ratios of choline (Ch) and acetylcholine (ACh) utilizing 50 nmoles of butyrylcholine (BCh) as internal standard. Insert shows the lower limit of sensitivity of the method (n = 6).

Fig. 2B represents an example of 50 mg of brain tissue taken through the method starting with the homogenization step. The sensitivity of the assay for acetylcholine in brain tissue is approximately 0.5 ± 0.07 nmoles (S.E.). Concentrations in whole brain tissue are 21.90 ± 1.36 nmoles/g (S.E.) for acetylcholine, and 62.15 ± 2.47 nmoles/g (S.E.) for choline (n = 6). This compares favorably with published reports in the literature in which whole brain levels were obtained without the use of microwave irradiation [6, 10].

Improvements in the assay of acetylcholine and choline have lagged slightly behind improvements in the detection devices. The sensitivity, selectivity and quantitative advantages obtained with a gas chromatograph—mass spectrometer tended to compensate for assay deficiencies. It was the purpose of this research to improve the assay to the point where it could reliably be used with a gas chromatograph equipped with a flame ionization detector. The method as used has an approximately 500% cost advantage over the initial purchase price of a gas chromatograph—mass spectrometer. In addition, a gas chromatograph costs much less to operate and requires less expertise. The column selected utilizes commonly available materials. The use of chloroform as the final injection solvent decreases variability over the more volatile dichloromethane required by the mass spectrometer. Consistency has also been enhanced by distillation of the acetone. For the first time, recovery through the method

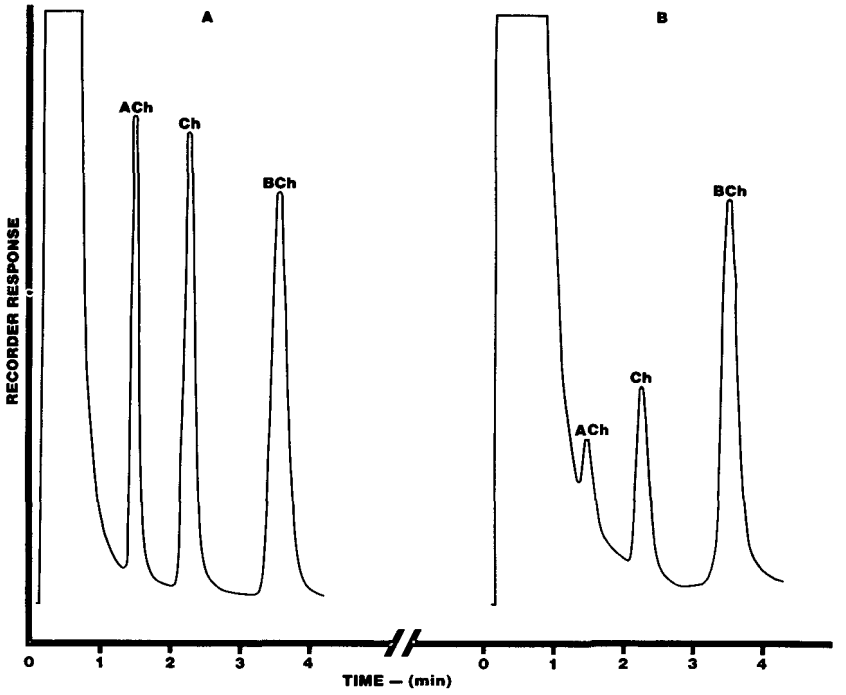


Fig. 2. Chromatogram of a 50-nmole equimolar mixture of acetylcholine (ACh), choline (Ch) and butyrylcholine (BCh) (A), and of a 50-mg rat brain extract (B). Two microliters of the chloroform layer were injected in (A) and (B). Attenuator settings: (A) 100 × 8; (B), 100 × 1. The levels of acetylcholine and choline in (B) were 1.09 ± 0.07 nmoles and 3.94 ± 0.16 nmoles (± S.E.), respectively (n = 7).

(80%) has been quantitated by the use of 4-dimethylamino-3-methyl-2-butanone. This assay and column should be readily adaptable to a nitrogen detector [5] or a stream splitter fitted to the gas chromatograph. These modifications will increase the sensitivity of the assay as well as allowing for the analysis of radioactive choline and acetylcholine.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the generous support of Dean Julian H. Fincher of the College of Pharmacy and the Research and Productive Scholarship support of the University of South Carolina.

REFERENCES

1 I. Hanin (Editor), Choline and Acetylcholine: Handbook of Chemical Assay Methods, Raven Press, New York, 1974.
2 J.J. Freeman and D.J. Jenden, The Source of Choline for Acetylcholine Synthesis in Brain (Minireview), Life Sci., 19 (1976) 949.
3 S.G. Butcher, L.L. Butcher, M.S. Harms and D.J. Jenden, J. Microwave Power, 11 (1976) 1.
4 D.J. Jenden, M. Roch and R.A. Booth, Anal. Biochem., 55 (1973) 438.

5 H. Kilbinger, J. Neurochem., 21 (1973) 431.
6 I. Hanin and D.J. Jenden, Biochem. Pharmacol., 18 (1969) 837.
7 G.G. Hammar, I. Hanin, B. Holmstedt, R.J. Kitz, D.J. Jenden and B. Karlen, Nature (London), 220 (1968) 915.
8 D.E. Schmidt, P.I.A. Szilagyi and J.P. Green, J. Chromatogr. Sci., 7 (1969) 248.
9 J.J. Freeman, R.L. Choi and D.J. Jenden, J. Neurochem., 24 (1975) 729.
10 K. Dross and H. Kewitz, Naunyn-Schmiedeberg's Arch. Pharmakol., 274 (1972) 91.

Journal of Chromatography, 163 (1979) 212—216
Biomedical Applications

CHROMBIO. 321

Note

High-performance liquid chromatographic assay of creatinine in human urine

DOUGLAS N. BUCHANAN*, SANDRA K. TAIT and EDWARD F. DOMINO

Department of Pharmacology, Lafayette Clinic, 951 E. Lafayette, Detroit, Mich. 48207 (U.S.A.)

(First received October 19th, 1978; revised manuscript received February 1st, 1979)

For several years we have been interested in the urinary concentrations of neurotransmitters and their metabolites in mental disease as a means of improving diagnosis and treatment. The most common way of reporting the 24-hour urinary concentration of neurotransmitters and their metabolites has been to express the value per milligram of creatinine. Thus, in order to obtain accurate ratios, a selective and accurate creatinine assay is imperative. The classical Jaffé alkaline picrate method [1], although rapid, has been reported [2] to be influenced by the presence of various organic compounds in the urine. Thus, the alkaline picrate creatinine determination does not possess the selectivity and the accuracy that we desired. By combining cation-exchange chromatography for the separation of creatinine from interfering, picrate-positive, compounds in urine and quantitation of the isolated creatinine by the alkaline picrate method, the accuracy of the determination can be improved [2]. This procedure has been automated [3] and represents a highly precise and quick means for the determination of urinary creatinine. However, the accuracy of this procedure has been reported [2—4] to be only slightly better than the classical Jaffé method. The pH control, the temperature control, and the precise timing of the alkaline picrate procedure [2] reduces the accuracy of the method. Some alternative means for the quantitation of creatinine after it is removed from the cation-exchange column was necessary.

The ability of a reversed-phase high-performance liquid chromatographic (HPLC) column to separate polar molecules in an aqueous medium appeared ideally suited for the analysis of creatinine. Creatinine, because of its inherent ultraviolet absorbance, could easily be detected at 254 nm [5]. We modified the HPLC creatinine procedure of Lim et al. [5] through the addition of an internal standard, 4-aminomethylpyridine (4-AMP). We report our results in this communication.

*To whom correspondence should be addressed. Reprint requests should be addressed to: Reprint Secretary, Lafayette Clinic, 951 E. Lafayette, Detroit, Mich. 48207, U.S.A.

EXPERIMENTAL

Materials

A Beckman DB ultraviolet—visible spectrometer at 515 nm was employed for the alkaline picrate creatinine analysis. An LC-50 high-performance liquid chromatograph (Bioanalytical Systems) with a μBondapak C_{18} (10 μm, Waters Assoc., Milford, Mass., U.S.A.) reversed-phase analytical column (30 cm × 3.9 mm I.D.) was used for the separation of creatinine and the internal standard. Detection was facilitated by a fixed wavelength (254 nm) ultraviolet detector (Altex, Model 153 analytical detector). A Speed Vac concentrator (Savant) was connected to an automatic freeze-dryer (Virtis, Model 10-010) for the concentration of aqueous samples.

4-Aminomethylpyridine (4-AMP), 99% pure, was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and was used without further purification. Creatinine zinc chloride was obtained from Eastman Chemicals (Rochester, N.Y., U.S.A.) and was used without further purification.

Solutions

Water was glass-distilled from an alkaline permanganate solution and was passed through a 0.22 μm Millipore filter before being used.

Standard creatinine solution. Creatinine zinc chloride was dissolved in 0.1 *N* HCl to afford a 1.41 mg/ml solution.

Internal standard solution. 4-Aminomethylpyridine was dissolved in water to afford a 0.98 mg/ml solution.

Standard urine. A 24-hour urine pool from a normal, healthy male was used in all analyses.

Citric acid buffer (pH 3.0). To 40 mmole of citric acid and 20 mmole of Na_2HPO_4 was added 1.0 l of water. The pH of this solution is 3.0 without adjustment.

Mobile phase. A 0.5 *M* sodium acetate solution (pH 4.7) containing 10% (v/v) methanol (glass-distilled) was prepared and was filtered through a 0.22-μm filter prior to use.

Analytical procedure

Preparation of creatinine standards. Solution 1: 0.50 ml of standard urine. Solution 2: 0.50 ml of standard urine + 0.20 ml of standard creatinine solution. Solution 3: 0.50 ml of standard urine + 0.40 ml of standard creatinine solution. Solution 4: 0.50 ml of standard urine + 0.60 ml of standard creatinine solution.

Sample preparation [5]. To each creatinine standard solution and to each 0.50-ml urine sample was added 0.50 ml of the internal standard solution. To each solution were added 24.0 ml of water and 5.0 ml of citric acid buffer (pH 3.0). Duplicate 3.00 ml aliquots were taken from each solution for creatinine analysis.

Cation-exchange chromatography. Fresh AG 50W-X12 (Bio-Rad Labs., Richmond, Calif., U.S.A.) cation-exchange resin, 100 mg per sample, was added to 2.50 ml of a 2.5 *M* NaOH solution and was allowed to sit overnight at ambient temperature. Resin was added to a Pasteur pipette (5 in.) that was lightly plugged with glass wool. The sodium hydroxide solution was allowed

to drain completely through the resin. Each column was washed with 2.0 ml of water. Each column was activated by the passage of 4.0 ml of citric acid buffer (pH 3.0) through the column. To each column were added 3.0 ml of the appropriate sample solution. Each column was allowed to drain, and was washed with 4.0 ml of water. Creatinine and 4-AMP were eluted from the resin by the addition of 4.0 ml of 1.0 *M* sodium acetate to the column. The sodium acetate eluate was collected in a small test-tube and was concentrated under vacuum to approximately 0.5 ml in a Speed Vac concentrator. To each sample was added 1.0 ml of water.

HPLC analysis

A 20-μl loop injector valve was used to place each sample onto a μBondapak C_{18} (10 μm) analytical column. The flow-rate of the mobile phase through the column was 1.0 ml/min. Detection was effected by a fixed wavelength (254 nm) ultraviolet detector. Creatinine eluted from the column at 4.5 min while 4-AMP eluted at 5.5 min (see Fig. 1).

Quantitation

Using the creatinine/4-AMP peak height ratio from the standard solutions and plotting this ratio against the creatinine concentration (mg/ml) added

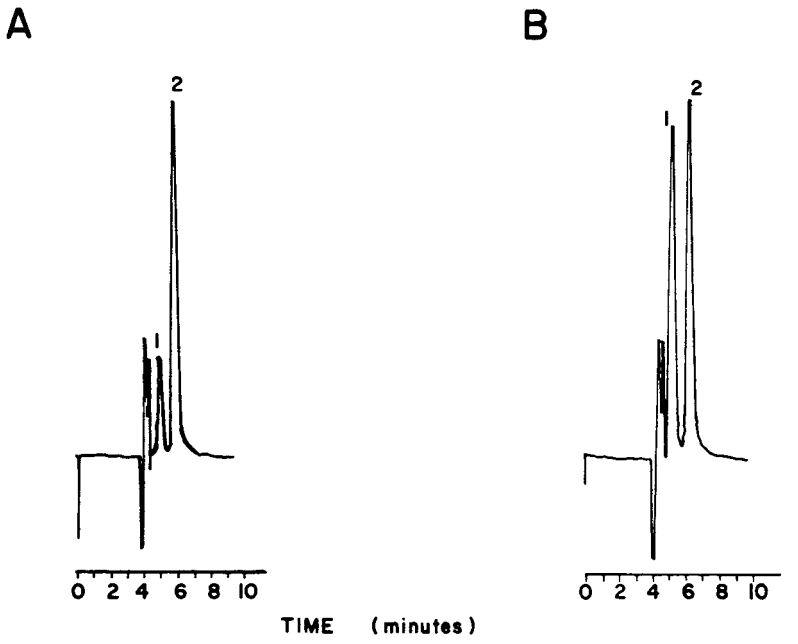


Fig. 1. Chromatograms of urinary creatinine. After addition of internal standard and passage through an ion-exchange column, the chromatograms obtained are shown for two different urinary concentrations of creatinine: (A) 0.83 mg/ml urine; (B) 2.57 mg/ml urine. 1 = Creatinine, 2 = internal standard, 4-aminomethylpyridine (4-AMP). Conditions: column, μBondapak C_{18} (10 μm); solvent, 0.5 *M* sodium acetate (pH 4.7) plus 10% methanol; flow-rate, 1.0 ml/min; detection at 254 nm.

to the standard urine, a least-squares line, $y = 0.35x + 0.33$, was fitted through the data points, correlation coefficient 0.993. The slope of the least-squares line was used for calculation of the creatinine concentration in the unknown urine samples.

Because of the relatively high urinary creatinine concentration in the 0.50—4.00 mg/ml range, the limit of detection by this method was not determined. However, this procedure afforded linearity over the range 0.02—6.15 mg/ml.

RESULTS AND DISCUSSION

The procedure of Lim et al [5] involved the separation of creatinine from urine by cation-exchange chromatography, elution of the creatinine from the column and HPLC reserved-phase analysis of the eluted creatinine with ultraviolet detection at 254 nm. Lim et al. [5] did not use an internal standard in this procedure.

We found that 4-AMP fits all of the qualifications of an acceptable internal standard [6]. Using a mobile phase of 0.5 *M* sodium acetate (pH 4.7) containing 10% (v/v) methanol at a flow-rate of 1.0 ml/min, creatinine had a retention time of 4.5 min while 4-AMP had a retention time of 5.5 min on a μBondapak C_{18} (10 μm) analytical HPLC column (Fig. 1).

Quantitative elution of both creatinine and 4-AMP from the cation-exchange resin was not effected through the use of 3.0 ml of 0.5 *M* sodium actetate as suggested by Lim et al. [5]. We found that 4.0 ml of 1 *M* sodium acetate afforded the quantitative elution of both creatinine and 4-AMP from the resin.

Using this HPLC method, we obtained creatinine concentrations from 24-hour urines (47 samples) that were an average of 14% lower than the creatinine concentrations obtained from these same 24-hour urine samples using the alkaline picrate method [7]. This result was not unexpected due to all of the variables that influence the results of the alkaline picrate method [2]. In a recent communication Chiou et al [4], using a cation-exchange HPLC column for the separation of creatinine from interfering plasma components and ultraviolet detection, reported plasma creatinine concentrations that were an average of 14.55% lower than the creatinine concentrations obtained from the same plasma samples using a SAM-6 Auto Analyzer. The automated creatinine analyzer utilizes the alkaline picrate method [2, 3].

Table I shows the accuracy of this HPLC method and the alkaline picrate method in the analysis of aqueous creatinine solutions. Both methods have about the same accuracy. It can be concluded that endogenous "quasi-creatinine" [2] compounds in the urine are responsible for the elevated urinary creatinine concentrations obtained by the alkaline picrate method.

The linearity of the HPLC method was investigated over the range 0.40—4.00 mg creatinine per ml urine, with a correlation coefficient of 0.970. Because of the relatively high urine creatinine concentrations (mg/ml range) the lower limit of sensitivity of this procedure was not determined. Repetitive analysis (nine times) of a single urine sample from a normal, healthy male afforded an average creatinine/4-AMP peak height ratio of 0.82 ± 0.06 (mean ± S.D.). The standard deviation is about 7% of the mean.

TABLE I

A COMPARISON OF THE HPLC METHOD AND THE ALKALINE PICRATE METHOD USING AQUEOUS CREATININE SOLUTIONS

Actual concentration (mg/ml)*	HPLC creatinine (mg/ml)**	Alkaline picrate creatinine (mg/ml)**
1.71	1.68	1.53
1.20	1.14	1.11
1.37	1.50	1.30
1.03	1.00	1.00

*Aqueous creatinine solutions prepared by colleague and analyzed "blind" by both procedures.
**Duplicate determinations with the average value recorded.

When 24-hour urinary indoleacetic acid (IAA) concentrations, expressed as mg IAA per mg creatinine, for normal control patients and for schizophrenic patients were based upon creatinine concentrations obtained by the alkaline picrate method [7], the IAA concentration for schizophrenic patients was significantly higher, at the 0.007 level, than for the control patients. When this same ratio was based upon creatinine values obtained from the HPLC procedure, the IAA concentration for schizophrenic patients was significantly higher (at the 0.001 level) than for the control patients [8].

REFERENCES

1 M. Jaffé, Hoppe-Seyler's Z. Physiol. Chem., 10 (1886) 391.
2 J.G.H. Cook, Ann. Clin. Biochem., 12 (1975) 219.
3 W.L. White, M.M. Erickson and S.C. Stevens, Chemistry for Medical Technologists, 3rd ed., C.V. Mosby Company, St. Louis, Mo., 1970, p. 84.
4 W.L. Chiou, G.W. Peng, M.A.F. Gadalla and S.T. Nerenberg, J. Pharm. Sci., 67 (1978) 292.
5 C.K. Lim, W. Richmond, D.P. Robinson and S.S. Brown, J. Chromatogr., 145 (1978) 41.
6 P.R. Brown, High Pressure Liquid Chromatography: Biochemical and Biomedical Applications, Academic Press, New York, 1973, p. 123.
7 J.D. Bauer, G. Toro and P.G. Ackerman, Bray's Clinical Laboratory Methods, 6th ed., C.V. Mosby Company, St. Louis, Mo., 1962, p. 65.
8 E.F. Domino, D.N. Buchanan, B. Mathews and S. Tait, unpublished results.

Journal of Chromatography, 163 (1979) 217—220
Biomedical Applications

CHROMBIO. 312

Note

Sensitive gas chromatographic assay of tinidazole in tissue and plasma

H. LAUFEN, F. SCHARPF and G. BARTSCH

Research & Development, Department of Pharmacology, Heinrich Mack Nachf., Illertissen/Bayern (G.F.R.)

(Received November 3rd, 1978)

Tinidazole (1-[2-(ethylsulfonyl)-ethyl]-2-methyl-5-nitroimidazole) is a synthetic antimicrobial agent used in the therapy of trichomoniasis in man. For the in vivo assay of tinidazole, methods have been described based on microbiological [1] and polarographic [2] measurements as well as thin-layer chromatography (TLC) with fluorescence detection [3] and high-performance liquid chromatography (HPLC) with spectrophotometric detection [4]. The sensitivity of these methods is about 0.2—0.5 μg/ml, which has proven to be satisfactory for the determination of therapeutic plasma levels of tinidazole in animal and in man [1—4].

Pharmacokinetic studies on the absorption and distribution of tinidazole at the sites of potential action, e.g. muscle and organ tissue, however, require a more sensitive method. This paper describes a sensitive and selective gas chromatographic (GC) assay which allows the measurement of concentrations of tinidazole in tissue to a lower limit of 50 ng/g. The method is also suitable for determination of tinidazole plasma concentrations.

EXPERIMENTAL

Standards and reagents

Tinidazole was supplied by Pfizer (Karlsruhe, G.F.R.), and 4-nitrodiphenylamine by Aldrich (Milwaukee, Wisc., U.S.A.). Dichloromethane, methanol, acetone, sodium sulphate and sodium hydroxide were obtained from Merck (Darmstadt, G.F.R.). All reagents were of analytical grade.

Extraction procedure

To 0.5 g of tissue homogenate in a 20-ml round-bottom centrifuge tube 50 μl of 6 *N* sodium hydroxide solution and 10 ml of a dichloromethane—metha-

nol (98:2) mixture were added. The organic solvent had been previously spiked with 10 μl of a solution of 4-nitrodiphenylamine in dichloromethane corresponding to 1 μg internal standard. The tube was shaken vigorously for 7 min and centrifuged at 2000 *g* for 5 min. The organic layer was transferred to a second centrifuge tube containing 500 mg powdered sodium sulphate, the mixture was then shaken for 5 min. After centrifugation, the organic phase was decanted into a tapered tube and reduced to approx. 1 ml under a stream of purified nitrogen. The cooled solution was kept at 4° for approx. 10 min. The liquid phase was then separated from the precipitates by pipetting it into a small reaction vessel and evaporating to dryness. The residue, dissolved in 50 μl acetone was shaken on a vibrator for 5 sec.

For the extraction of tinidazole from plasma the procedure was slightly modified: The internal standard was added directly to the plasma and the step of cooling the extracts was omitted, because no precipitate was formed.

Gas chromatographic assay

The gas chromatograph used was a Becker Packard Model 419 equipped with a PN detector (Perking Elmer). Integration of the chromatographic signals was performed by a digital integrator (Spectra Physics Model SP 4000).

Using a 5-μl Hamilton microsyringe, 0.2—1 μl of the acetone solution was injected under GC conditions: analytical glass column (1 m × 2 mm I.D.) packed with 3% OV-11 on Gas-Chrom Q (80—100 mesh) (WGA, Düsseldorf, G.F.R.); carrier gas, nitrogen at a flow-rate of 20 ml/min; air and hydrogen flow-rates, 200 ml/min and 1.5 ml/min, respectively; column temperature, 215°; injection port and detector temperature 245°.

RESULTS AND DISCUSSION

Under the conditions described, integrable peaks of tinidazole (retention time 225 sec) and 4-nitrodiphenylamine (retention time 305 sec) were obtained. Chromatograms from tinidazole assays in tissue and plasma are shown in Fig. 1. Calibration curves were plotted after adding suitable amounts of tinidazole to human plasma or rat tissues. The linearity of the method was established for ranges of 0.1—50 μg tinidazole per ml plasma and 0.05—0.5 μg tinidazole per g tissue.

The repoducibility of six consecutive analyses of plasma to which 0.5 μg/ml tinidazole had been added, was ± 4.1% (coefficient of variation), corresponding to an analytical value of 0.5 ± 0.021 μg/ml. The lower limit of quantitation was 50 ng tinidazole per g tissue homogenate. Although tinidazole in plasma could be detected to a lower limit of 20 ng/ml, there was no need to confirm the feasibility of this method for quantification of plasma in the lower nanogram range, because therapeutic tinidazole concentrations in plasma are in a far higher range. The yield of the assay was determined at concentrations of 500 ng/ml plasma by successive injections of plasma extract containing tinidazole and the respective standard solution. For tinidazole, a yield of 76.5 ± 3.2% was obtained. The yield of the internal standard was 53.9 ± 3.1%. These yields cover the entire analytical procedure up to the injection of the sample into the gas chromatograph.

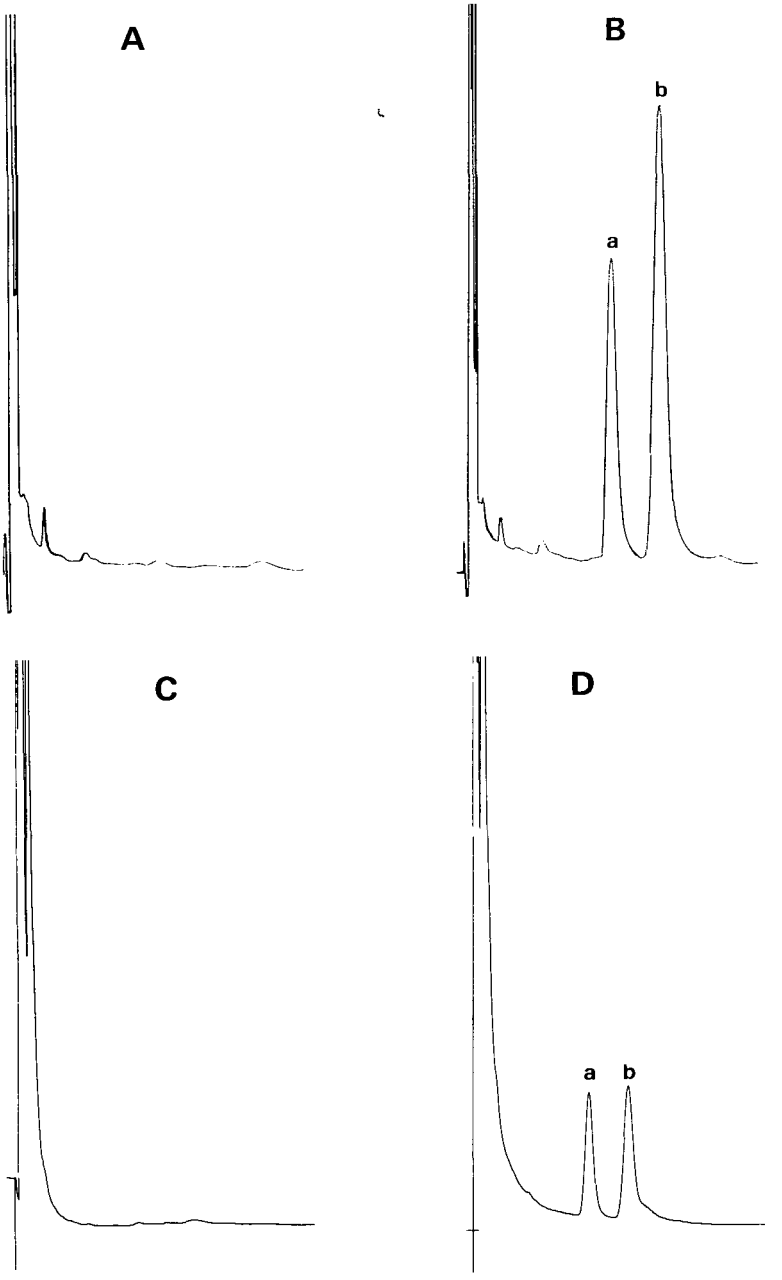


Fig. 1. Chromatograms of plasma and tissue extracts. (A) Blank plasma; (B) plasma spiked with tinidazole (a) and 4-nitrodiphenylamine (b); (C) intestinal tissue blank; (D) intestinal tissue spiked with tinidazole (a) and 4-nitrodiphenylamine (b).

This method has been applied to the quantitative analysis of tinidazole in tissue and plasma of humans and rats. The application of the same method of assay to several biological materials with a widespread range of tinidazole concentrations enhances the use of this analytical procedure.

REFERENCES

1 T. Ripa, L. Weström, P.-A. Mårdh and K.-E. Andersson, Chemotherapy, 23 (1977) 227.
2 J.A. Taylor, Jr., J.R. Migliardi and M. Schach von Wittenau, Antimicrob. Ag. Chemother., (1969) 267.
3 P.G. Welling and A.M. Monro, Arzneim.-Forsch., 22 (1972) 2128.
4 J. Nachbaur and H. Joly, J. Chromatogr., 145 (1978) 325.

Journal of Chromatography, 163 (1979) 221—224
Biomedical Applications

CHROMBIO. 320

Note

Detection of tuberculostearic acid in mycobacteria and nocardiae by gas chromatography and mass spectrometry using selected ion monitoring

LENNART LARSSON

Department of Technical Analytical Chemistry, University of Lund, S-220 07 Lund (Sweden)

PER-ANDERS MÅRDH

Department of Medical Microbiology, University of Lund, S-223 62 Lund (Sweden)

and

GÖRAN ODHAM

Laboratory of Ecological Chemistry, University of Lund, S-223 62 Lund (Sweden)

(Received October 18th, 1978)

Selected ion monitoring means the focusing of a mass spectrometer on fragments specific for the particular compound under study. When combined with gas chromatography (GC), the technique offers a very high degree of selectivity and sensitivity for the quantitative analysis of organic compounds which are volatile or can be converted into volatile derivatives. Many microorganisms produce such cellular or extracellular compounds, specific for a given genus or species [1, 2].

Tuberculosis is still one of the most important infectious diseases of mankind, the microbial diagnosis of which has in point of principle remained unchanged since the discovery of the causative organism. Thus, the isolation of *Mycobacterium tuberculosis* and several other pathogenic mycobacteria from clinical specimens takes several weeks. Mycobacteria, and also a limited number of other organisms of the order *Actinomycetales*, contain appreciable amounts of 10-methyloctadecanoic acid, mainly in the form of phospholipids in the cell wall. Because it is considered unique for these microorganisms, this acid is usually referred to as tuberculostearic acid [3—7].

In the present investigation we assessed whether selected ion monitoring of tuberculostearic acid could be used to detect mycobacteria, and thus, by

extension, be of use for the rapid diagnosis of mycobacterial infections.

Freshly isolated strains of *M. tuberculosis* were cultured in Proskauer—Beck medium, autoclaved, lyophilized and extracted with chloroform—methanol. The supernatants were then evaporated, methanolysed, and injected onto the GC column. By mass spectrometry (MS) it was demonstrated that one of the chromatographic peaks present in each chromatogram obtained represented the methyl ester of 10-methyloctadecanoic acid, the structure of which was confirmed by a comparative GC—MS study of the authentic compound, synthesized in our laboratory. The mass spectrum gives characteristic peaks, for instance at *m/e* 167 (loss of $CH_3(CH_2)_7$ followed by CH_3OH) and *m/e* 312 (molecular weight, M) (Fig. 1).

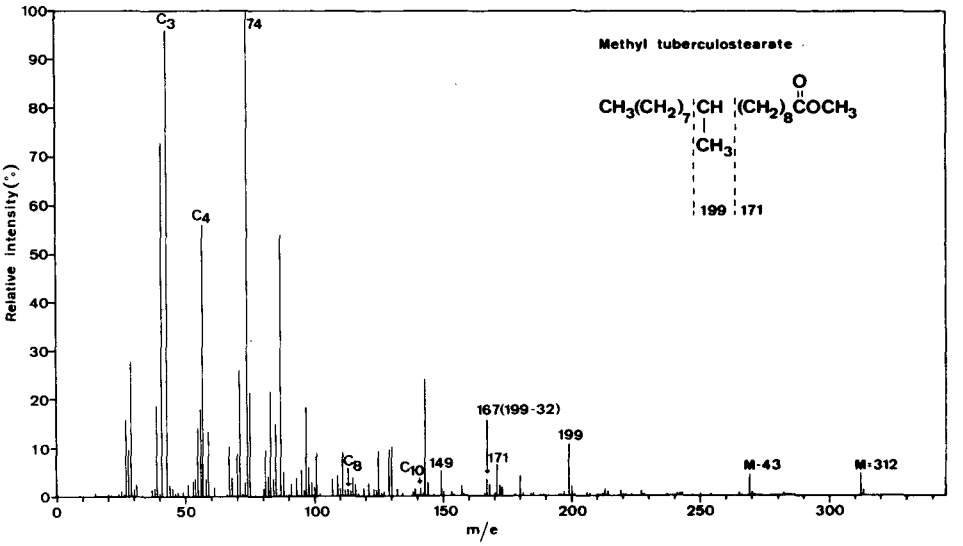


Fig. 1. Mass spectrum, in which characteristic fragments are indicated, of methyl tuberculostearate obtained from *Mycobacterium tuberculosis*. The bacteria had been cultured on Proskauer—Beck medium (Difco) at 37° for 30 days, autoclaved (121°, 60 min, 1.1 kP/cm²), washed with distilled water and lyophilized. Of the lyophilized cells, 2 mg were extracted with 1 ml of chloroform—methanol (2:1, v/v) overnight, after which the supernatant was evaporated to dryness under a stream of nitrogen, methanolysed for 20 h at 80° using 3% methanolic HCl, evaporated, and taken up in 100 µl of *n*-hexane. One microlitre was injected into a 2 m × 2 mm I.D. glass column packed with 3% OV-101 on Chromosorb W HP (80—100 mesh). The helium carrier gas flow-rate was 25 ml/min, and the column temperature 230°. A Varian MAT mass spectrometer was used, employing an ion source temperature of 230° and an electron energy of 70 eV.

The chromatograms obtained when analysing the extracts by monitoring at *m/e* 167 and *m/e* 312 contained one single peak, that of methyl 10-methyloctadecanoate. The greatest sensitivity was achieved at *m/e* 312, by which the fatty acid ester could be detected in as little as 2 ng of lyophilized cells, corresponding to 20 pg of ester. When employing ion selection at *m/e* 167, the signal-to-noise ratio was less favourable and the sensitivity decreased by a factor of approximately five.

M. tuberculosis, cultured on slants of Löwenstein—Jensen medium, was also

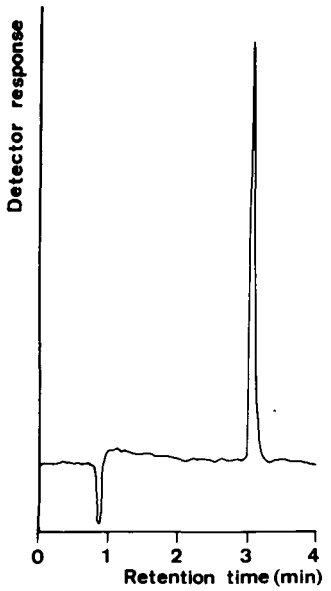


Fig. 2. Chromatogram from an extract of a culture of *Mycobacterium tuberculosis* obtained by selected ion monitoring at *m/e* 312. The bacteria had been collected by washing Löwenstein—Jensen slants with saline after five days of incubation at 37°. The preparation of the sample and the GC conditions were identical with those used in Fig. 1. Of a final solution of 100 µl of *n*-hexane, 0.1 µl was used for analysis. The peak represents approximately 200 pg of methyl tuberculostearate. The negative peak represents a pressure increase in the mass spectrometer caused by injection of the solvent.

tested. After 5 days of incubation at 37°, when still no colonies of mycobacteria could be detected by the naked eye, the slants were washed with saline and the suspensions so obtained treated as described above. A chromatogram using ion selection at *m/e* 312 is shown in Fig. 2. On duplicate cultures, mycobacterial colonies could first be visually observed 14—20 days after inoculation.

The specificity of the technique, when monitoring at *m/e* 167 and *m/e* 312, was evaluated in analyses of *M. africanum*, *M. avium*, *M. bovis* (Strain Bacillus Calmette-Guerin [BCG]), *M. kansasii*, *M. smegmatis*, *M. tuberculosis*, *Nocardia asteroides*, *N. brasiliensis* and *N. rubra*. In addition, *Escherichia coli* and strains of pneumococci, staphylococci, streptococci, and anaerobes, such as organisms of genera *Bacteroides*, *Clostridium* and *Propionibacterium*, were analysed. The chromatograms representing the mycobacteria and *Nocardia* sp. contained one peak, while those of the other bacteria studied were completely blank.

The GC—MS technique described provides chromatograms containing one single peak which are consequently easy to interpret. The sensitivity for tuberculostearic acid was found to be approximately 50 times greater than that obtainable with the flame ionization detector. Since many microbial products are unique for a particular genus or species, selected ion monitoring constitutes a diagnostic tool having great potential and wide scope for use within the field of clinical microbiology. The expense of mass spectrometry is a draw-back but should be related to the obvious advantages offered by the technique, such as a means for the rapid diagnosis of tuberculosis.

ACKNOWLEDGEMENT

This study was supported by a grant from the Swedish National Association Against Heart and Chest Diseases.

REFERENCES

1 Y. Henis, J.R. Gould and M. Alexander, Appl. Microbiol., 14 (1966) 513.
2 N. Shaw, Advan. Appl. Microbiol., 17 (1974) 63.
3 C. Bordet and G. Michel, Biochim. Biophys. Acta, 70 (1963) 613.
4 H. Okuyama, T. Kankura and S. Nojima, J. Biochem., 61 (1967) 732.
5 C.O. Thoen, A.G. Karlson and R.D. Ellefson, Appl. Microbiol., 21 (1971) 628.
6 C.O. Thoen, A.G. Karlson and R.D. Ellefson, Appl. Microbiol., 22 (1971) 560.
7 R.W. Walker, H. Barakat and J.G.C. Hung, Lipids, 5 (1970) 684.

Journal of Chromatography, 163 (1979) 225—229
Biomedical Applications

CHROMBIO. 337

Note

High-performance liquid chromatographic determination of ascorbic acid in urine

Effect on urinary excretion profiles after oral and intravenous administration of vitamin C

EUGENE S. WAGNER, BARRY LINDLEY and ROBERT D. COFFIN*

Department of Chemistry and Muncie Center for Medical Education, Ball State University, Muncie, Ind. 47306 (U.S.A.)

(First received November 14th, 1978; revised manuscript received March 2nd, 1979)

Methods for analyzing the ascorbic acid content of biological fluids such as urine have traditionally been titrimetric [1] and/or spectrophotometric [2]. These techniques, although quite accurate, are laborious and time-consuming. More recently, high-performance liquid chromatography (HPLC) has been applied to the measurement of ascorbic acid in foods, multi-vitamin products, and biological fluids [3, 4]. We have used HPLC to determine the ascorbic acid content of urine samples, which is more accurate than the traditional methods of analysis and which enables one to obtain a direct readout of the amount of ascorbic acid in a sample within minutes. In the course of this investigation, urinary excretion profiles for orally ingested and intravenously infused doses of ascorbic acid have been determined and compared to examine the role the absorptive process plays on the reported [5] diminished urinary excretion of ascorbic acid over the 500-mg dosage.

EXPERIMENTAL

Materials

Ascorbic acid (1.0-g tablets), commercially obtained, were used by the nine subjects for the oral ingestion portion and a 10-ml ampoule of Cevalin, 1 g (Eli Lilly & Co., Indianapolis, Ind., U.S.A.), was used for the intravenous infusion portion of this experiment.

A standard ascorbic acid solution was prepared using 100 mg ascorbic acid

*Present address: Department of Pediatrics, Indiana University School of Medicine, Indianapolis, Ind. 46202, U.S.A.

(Fisher Scientific, Pittsburgh, Pa., U.S.A.) per liter 10% metaphosphoric acid (Fisher Scientific). The titration dye solution was prepared using 80 mg 2,6-dichlorophenol-indophenol (Sigma, St. Louis, Mo., U.S.A.) per 500 ml of solution containing 12 ml of phosphate buffer at pH 7.0.

The mobile phase of the HPLC analysis was 0.8% metaphosphoric acid prepared by adding 8 g metaphosphoric acid (Fisher Scientific) to double-distilled, Millipore-filtered water (1000 ml). This solution was de-aerated by vacuum.

Sample collection

Nine healthy subjects ingested 1.0 g of ascorbic acid for 13 days. On the 7th day each subject collected urine samples at each of the following designated times: before ingestion of the tablet and 3, 6, 9, 12, 15 and 24 h after ingestion of the tablet. On the 14th day each subject received a 1.0-g intravenous infusion of the vitamin and again collected urine samples at the times designated above. The volume of urine collected at each time interval was recorded, and the sample submitted for analysis was acidified with 10% metaphosphoric acid (5 volumes of urine plus 1 volume of acid).

Titrimetric procedure

Ascorbic acid present in the urine samples was determined by titration with the dye, 2,6-dichlorophenol-indophenol, solution. This method depends on the reducing power of ascorbic acid. Since other reducing substances may interfere with the determination of ascorbic acid, the titration was performed rapidly in acid solution. A 10-ml aliquot of the dye solution is titrated by the constant dropwise addition of the sample of urine which has been acidified (the dye solution is blue at the start but changes to deep red upon addition of the first few drops of acidified urine). The end point is indicated by the disappearance of the red color. All determinations were made in triplicate.

High-performance liquid chromatography

Apparatus and operating conditions. A Model ALC-202 liquid chromatograph equipped with a Model 6000 solvent delivery system (Waters Assoc., Milford, Mass., U.S.A.) was used in the study. The column effluents were monitored with the 254-nm detector. Samples were introduced into the system via syringe injection. The flow-rate was 3.0 ml/min.

Column. A 30 cm × 4 mm I.D. μBondapak C_{18} column (Waters Assoc.) was used. μBondapak has a monomolecular layer of octadecyltrichlorosilane chemically bonded to μPorasil beads having an average particle size of 10 μm. The number of theoretical plates, based upon ascorbic acid at 3.0 ml/min was 2300. The chromatographic system was flushed nightly with methanol, as recommend by the supplier, ensuring removal of lipophilic substances from the column.

Standard curve. Samples of ascorbic acid in 0.8% metaphosphoric acid were prepared to contain 0.50, 0.75, 1.00, and 1.50 mg of ascorbic acid per ml. Aliquots (5.0 μl) of these solutions were injected into the chromatographic system, and the resulting peak heights were plotted against concentration

for the calibration curve. Each point was the average of twelve injections. The reliability to the straight line was 0.993, the slope was 0.3448 and the y intercept was −0.0012 integrator units. These data indicate the procedure is amenable to use of a single-point standard.

Mobile phase. Metaphosphoric acid (0.8%) prepared with double-distilled, Millipore-filtered water was de-aerated by vacuum before use.

Sample preparation. Each urine sample was filtered with a sample clarification kit (Waters Assoc.) which can remove fine particles of 0.5 μm or greater. A 5-μl portion of the acidified urine sample was injected, the resulting peak height determined, and the quantity of ascorbic acid in the sample calculated by reference to the previously derived calibration curve. Knowing the total volume of urine excreted at each time allowed the calculation of the total amount of ascorbic acid at the time in question. All determinations were made in triplicate.

RESULTS AND DISCUSSION

Results of the determination of ascorbic acid in urine using a modification of the chromatographic procedure described by Sood et al. [3] were compared (Table I) with results of the 2,6-dichlorophenol-indophenol titration. The titration procedure was used for comparison because of the wide use of the method. The precision of the visual titration procedure is considerably reduced because of the subjectiveness of end point determinations.

The ideal mobile phase to determine ascorbic acid in urine is 0.8% metaphosphoric acid solution. Other mobile phases [including ammonium salts in methanol—water (50:50)] resulted in values for ascorbic acid that were too high by 6—12%. This was attributed to the presence of other substances

TABLE I

COMPARISON OF URINARY ASCORBIC ACID LEVELS OBTAINED BY THE TWO METHODS

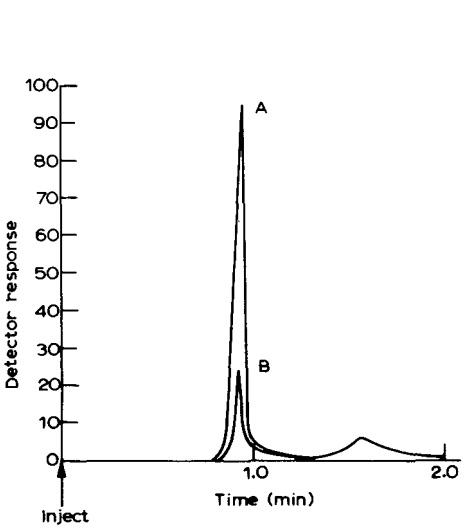
Urine sample No.*	Concentration of ascorbic acid (mg/ml) ± S.D.		
	Amount of ascorbic acid added to urine sample	Titrimetric method**	HPLC method**
1	2.0	1.1 ± 0.1	2.6 ± 0.1
2	20.0	15.2 ± 0.9	20.6 ± 0.1
3	35.0	34.2 ± 1.7	35.1 ± 0.2
4	62.5	61.1 ± 1.8	64.7 ± 0.2
5	62.5	62.8 ± 1.2	65.7 ± 0.5
6	20.0	20.3 ± 1.3	20.0 ± 0.1
7	10.0	9.8 ± 0.5	10.6 ± 0.1

*Urine sample spiked with ascorbic acid.
**Average of triplicates.

in urine (e.g., uric acid) which exhibit peaks corresponding to the retention volume of ascorbic acid. A representative chromatogram of ascorbic acid in urine using the 0.8% metaphosphoric acid solution mobile phase is presented in Fig. 1. A problem which was encountered with other mobile phases but not with 0.8% metaphosphoric acid solution was that when the chromatographic column had not been used for some time (ca. 45—60 min), the first sample injected always gave a value that was 7—14% low. Once the column had been pretreated with the first sample, quantitation of subsequent injections was precise. The use of 0.8% metaphosphoric acid solution as the mobile phase eliminated this phenomenon, with the result that the quantitation of all injections was accurate and precise no matter what the time interval between injections.

This liquid chromatographic method was used to determine the urinary excretion profiles for both orally ingested and intravenously infused 1.0-g doses of ascorbic acid. A plot of the mean urine concentrations of ascorbic acid after the oral dose and the intravenous infusion of ascorbic acid with time in 9 subjects (Fig. 2) showed that the peak of mean concentrations occurred at 3 h and 6 h for the infused and oral doses, respectively.

The data show that the urinary excretion of the orally ingested 1000-mg doses of vitamin C (610±65 mg) using 9 subjects is in agreement with our earlier study [5]. The results of this earlier study involving 24 healthy subjects receiving daily oral doses of 100, 250, and 500 mg ascorbic acid indicated that they excreted in their urine amounts of ascorbic acid equal to the amount



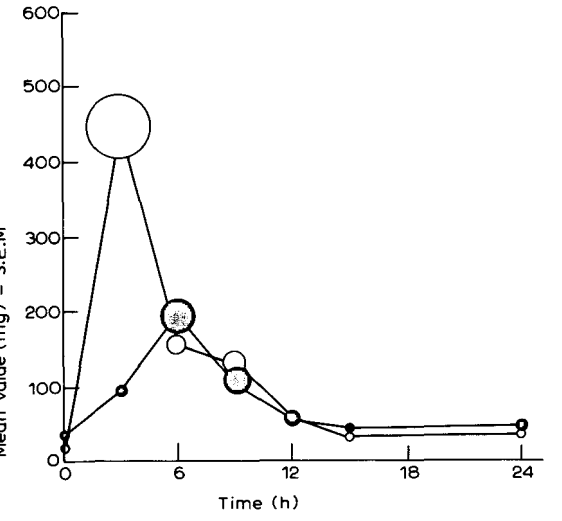


Fig. 1. HPLC chromatogram of: (A) 1.78 mg/ml ascorbic acid in urine; (B) 1:4 dilution of (A). Flow-rate, 3.0 ml/min; chart speed 2 in./min; range = 64.

Fig. 2. Mean±S.E.M. values of ascorbic acid excreted by 9 subjects in 24 h versus time after ingestion and infusion of 1.0 g of the vitamin. •, Oral ingestion; 610±65 mg excreted. ○, Intravenous infusion; 930±60 mg excreted.

ingested. With doses of 1.0 and 2.0 g per day, urinary excretion dropped to 54% and 36% of the administered dose, respectively. In addition, a dramatic increase in the urinary excretion of ascorbic acid (930±60 mg) is demonstrated when these same 9 subjects receive the vitamin via intravenous infusion. Consequently, if the route of administration of the vitamin is intravenous infusion rather than oral ingestion (by this means circumventing the intestinal absorption of the vitamin), over 90% of the infused dose is excreted in the urine. Therefore, large portions of single oral doses of ascorbic acid greater than 500 mg cannot be accounted for by urinary excretion because there is a decrease in the efficiency of ascorbic acid intestinal absorption at the higher dosage levels.

ACKNOWLEDGEMENTS

This study was supported by a Ball State University Faculty Research Grant. We wish to thank the nine first-year medical students in the Muncie Center for Medical Education who served as the subjects in this study. We are indebted to Dr. Douglas Triplett for his participation in the intravenous infusion portion of the experiment.

REFERENCES

1 L.J. Harris and S.N. Ray, Lancet, i (1935) 71.
2 W.T. Caraway, in N.W. Tietz (Editor), Fundamentals of Clinical Chemistry, Saunders, Philadelphia, Pa., 1970, pp. 175—176.
3 S.P. Sood, L.E. Sartori, D.P. Wittmer and W.G. Haney, Anal. Chem., 48 (1976) 796.
4 L.A. Pachla and P.T. Kissinger, Anal. Chem., 48 (1976) 364.
5 E.S. Wagner and K. Wagner, IRCS Medical Science, 3 (1975) 617.

Journal of Chromatography, 163 (1979) 230—235
Biomedical Applications

CHROMBIO. 338

Note

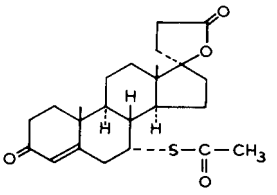
High-performance liquid chromatographic determination of canrenone, a major metabolite of spironolactone, in body fluids

GEORG B. NEURATH and DORIT AMBROSIUS

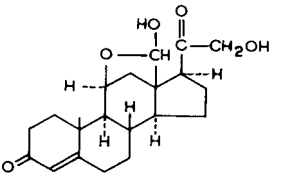
Institut für Biopharmazeutische Mikroanalytik, 2000 Hamburg 56 (G.F.R.)

(Received January 16th, 1979)

As a closely related compound, spironolactone (I) acts as a competitive inhibitor of aldosterone (II) in the exchange processes of the distal tubules of the kidney. Accordingly, the output of sodium is increased, and the output of potassium is decreased. The importance of spironolactone in the therapy of edematous conditions is thus explained.

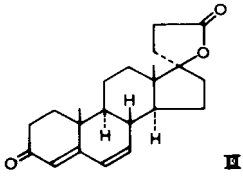


I



II

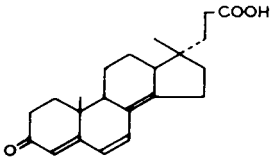
After oral administration, spironolactone is rapidly metabolized. The first step is the elimination of the 7α-acetylthio group. The des-acetylthio-Δ6 derivative formed is canrenone (III), first identified by Gochman and Gantt [1] in 1962, who at the same time described a highly sensitive fluorimetric method for the study of plasma levels of, as claimed by the authors, canrenone.



III

Their method consists of the extraction of the plasma with dichloromethane, re-extraction of the dichloromethane with 65% sulfuric acid, and measurement of the fluorescence (excitation 465 nm, emission at maximum 520 nm). These conditions are very similar to those used for the determination of endogenous cortisol and corticosterone in plasma first described by Sweat [2] and simplified in a large number of publications since then. The specificity of the fluorimetric method for this purpose has been critically examined by Pirke and Stamm [3].

The fluorimetric determination of canrenone has been used by many authors in recent years [4—6]. On reaction with sulfuric acid the fluorophor (IV) can be produced from canrenone and other metabolites, even those having an open ring.



IV

Canrenone is possibly the major effective principle of spironolactone. Funder et al. [7] have proved that the intact γ-lactone ring is necessary for the receptor reaction. Therefore, there exists an important need for a sensitive and specific method for the assay of canrenone.

This paper describes a precise, specific, and sensitive method for the determination of canrenone by means of high-performance liquid chromatography (HPLC) and compares it with the fluorimetric method.

EXPERIMENTAL

Apparatus

High-performance liquid chromatography was performed on a Pye-Unicam LC 20 separator equipped with a Pye-Unicam LC 3 ultraviolet (UV) absorbance detector (Philips, Kassel, G.F.R.) operated at 283 nm. A 25 cm × 0.25 cm I.D. glass column packed with LiChrosorb SI-100, 10 μm (Merck, Darmstadt, G.F.R.) or Nucleosil 100, 10 μm (Macherey-Nagel, Düren, G.F.R.) was used for separation.

Fluorimetric measurements were carried out on a Perkin-Elmer Filter Fluorimeter 1000 M in 3-ml quartz cuvettes (Bodenseewerk Perkin-Elmer, Überlingen, G.F.R.) equipped with a pulsed xenon lamp (6.25 W) and a 450-nm interference filter adjusted to 457 nm.

The mass spectrum of canrenone was measured using a Hewlett-Packard 5992 A computer-controlled gas chromatography—mass spectrometry system.

Materials

Canrenone. A 400-mg amount of potassium canrenoate (Boehringer, Mannheim, G.F.R.) was dissolved in about 5 ml of methanol, and glacial acetic acid

was added until the canrenoic acid had precipitated. The acid was filtered off, washed twice with 5 ml of methanol and dried by washing with 5 ml of *n*-hexane.

The dry acid was heated carefully until the visible evolution of water had ceased. The residue was recrystallized from 150 ml of ethyl acetate and dried (m.p. 161°; UV maximum 283 nm; mass spectrum *m/e* (%): 340 (M^+, 99), 267 (100), 91 (56), 136 (39), 325 (20), 227 (20)).

Solvents and reagents. All solvents and reagents used for HPLC and fluorescence determinations were of analytical grade. Dichloromethane for fluorescence measurements was of spectroscopic grade (Merck).

Serum. Serum for blanks and recovery studies was obtained from healthy volunteers.

Extraction

Serum. To 1 ml of serum, 200 μl of chloroform were added. The sample was vigorously shaken on a rotary mixer (Cenco, Breda, The Netherlands) for 30 min and then placed in a 3-ml reactivial (Pierce-Eurochemie, Rotterdam, The Netherlands) and centrifuged for 10—15 min at 5500 *g*. Aliquots of 50—100 μl of the chloroform solution were injected for HPLC.

Urine. To a 1-ml sample of urine, 0.5 ml of a β-glucuronidase solution (3000 Fishman units/ml)(Schering AG, Berlin, G.F.R.) in sodium acetate buffer (pH 4.6) was added and the mixture was incubated at 37° for 18 h. Chloroform (200 μl) was added and the sample was moderately shaken for 30 min, placed in a 3-ml reactivial and centrifuged for 10 min at 5500 *g*. 100 μl of the chloroform phase were injected for HPLC.

High-performance liquid chromatography

Serum. The mobile phase, chloroform—*n*-hexane (50:50), was pumped through the column at a flow-rate of 2.0 ml/min at a pressure of 7 MPa. The retention time of canrenone was 4.5 min.

Urine. The mobile phase was chloroform—*n*-hexane (55:45) at a flow-rate of 2.0 ml/min and pressure of 7 MPa. The retention time of canrenone was 4.0 min.

Sensitivity, accuracy, and precision of HPLC

A standard curve for canrenone was prepared by carrying out the analysis with test solutions and 31 repeated injections ranging from 1.25 to 20 ng canrenone. A Nucleosil 100, 10 μm column was used in these experiments.

The over-all average peak area and its standard deviation were found to be 5.53 mm^2/ng of canrenone, $s = \pm 0.33$. Linearity was obtained over the entire range.

The recovery rate was determined by carrying out the analysis on serum samples to which canrenone had been added at different concentrations ranging from 5 to 80 ng/ml. The average peak areas after the analytical procedure were 5.41 mm^2/ng canrenone ($s = \pm 0.27$), corresponding to a recovery of 97.8%.

The limit of the determination is about 5 ng/ml serum.

Fluorimetric determination

Canrenone standard. For the stock solution, 20 mg of canrenone were dissolved in 10 ml of ethanol and diluted to 1000 ml with water. Working standards were prepared daily by further dilution of the stock with water. Aliquots of the aqueous solution were added to 10 ml of dichloromethane. Four milliliters of 65% sulfuric acid were added, and the sample shaken for 30 sec. The acid phase was separated and kept at room temperature for 1 h.

At sensitivity × 1 the fluorescence values given in Table I were measured (excitation 457 nm, emission 520 nm).

TABLE I

FLUORESCENCE OF CANRENONE STANDARDS

Canrenone (ng)	7.5	15	30	60	90	120	150
Fluorescence	4	8	16	32	49	61	92

Spiked serum. To 0.2 ml serum and 5 ml water, aliquots of the standard canrenone solution were added. Dichloromethane (15 ml) was added and the sample shaken for 30 sec. The mixture was centrifuged, and the aqueous phase removed. To the dichloromethane, 1 ml of 0.1 *N* sodium hydroxide solution was added, the mixture shaken for 15 sec, and the aqueous phase discarded. Four milliliters of 65% sulfuric acid were added to 10 ml of the dichloromethane solution and shaken for 30 sec. The acid phase was separated and the fluorescence measured as described for the standard. The results are given in Table II.

TABLE II

FLUORESCENCE OF CANRENONE-SPIKED SERUM

Canrenone (ng) added to 0.2 ml serum	0	5	10	20	40	60	80
Fluorescence	0*	2	5	10	22	33	43

*Adjusted to zero.

Serum samples after administration of spironolactone. The samples were treated for fluorescence measurement as described for the spiked samples. A blank serum sample of each subject was spiked for comparison (subject A: 8 ng canrenone added to 0.2 ml serum — fluorescence value 3; B: 5 ng canrenone added to 0.2 ml serum — fluorescence value 2).

RESULTS

The high-performance liquid chromatography of canrenone gives precise, sensitive and reproducible determinations of this compound, which probably

represents the major active principle of spironolactone, without interference by inactive metabolites and endogenous corticosteroids. The method thus forms a useful tool for studies of the pharmacokinetics and bio-availability of spironolactone.

As a specific method, HPLC produces lower values than the fluorimetric determinations utilizing a dichloromethane-soluble fraction isolated from body fluids without further separation steps.

Peak serum values of canrenone determined by HPLC after oral administration of 100 mg spironolactone in different preparations assayed were of the order of magnitude of 45 ng/ml serum, as shown in Fig. 1.

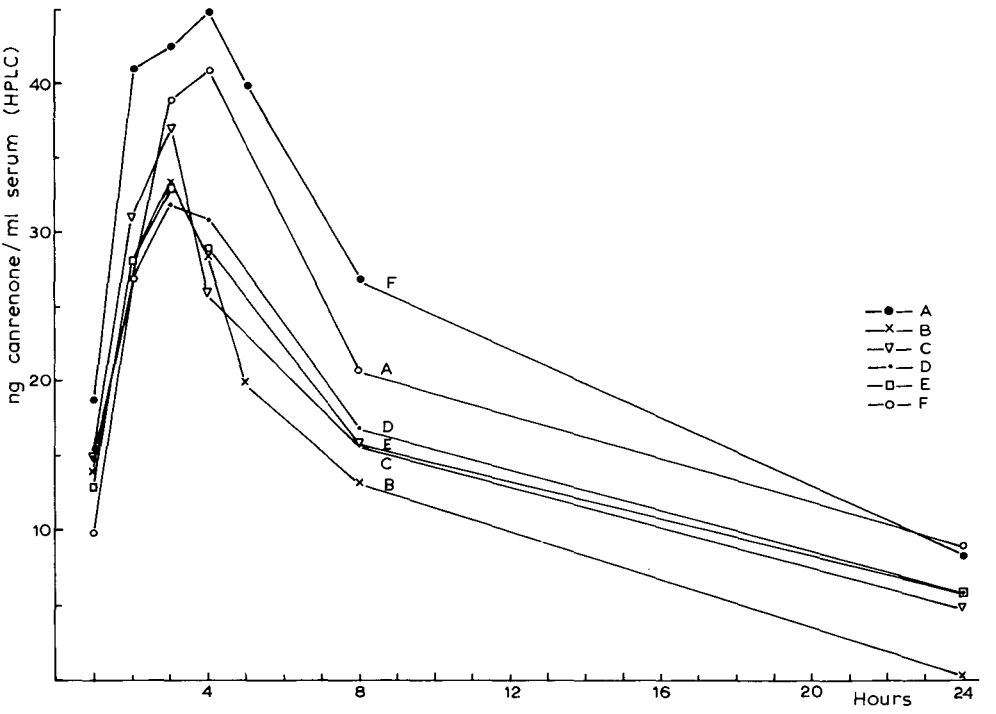


Fig. 1. Serum levels of canrenone after oral administration of 100 mg spironolactone in different preparations.

Literature data on the fluorimetric assay indicate peak values of 400—500 ng/ml serum for comparison. A comparison of HPLC and fluorimetric values collected on a small number of serum samples from two male subjects documents the difference, as shown in Table III.

TABLE III

COMPARISON OF SERUM LEVELS OF CANRENONE DETERMINED BY MEANS OF HPLC AND FLUORIMETRY

	Hours after oral administration of spironolactone	HPLC (ng canrenone per ml serum)	Fluorimetry* (ng fluorigenic compounds as "canrenone" per ml serum)
Male subject A	blank	0	+
	1	10	72.5
	2	31	320.0
	3	39	422.5
	4	45	282.5
	6	34	207.5
	8	24	80.0
Male subject B	blank	0	+
	1	8	10.0
	2	11	125.0
	3	18	217.5
	4	21	282.5
	6	17	150.0
	8	14	132.5

*+ = Blank of the order of magnitude of 25 ng "canrenone" per ml serum, adjusted to zero.

ACKNOWLEDGEMENT

The authors wish to thank the Wissenschaftliche Leitung, Boehringer-Mannheim GmbH, G.F.R., for the sample of canrenoic acid.

REFERENCES

1 N. Gochman and C.L. Gantt, J. Pharmacol. Exp. Ther., 135 (1962) 312.
2 M.L. Sweat, Anal. Chem., 26 (1954) 773.
3 K.M. Pirke and D. Stamm, Z. Klin. Chem. Klin. Biochem., 10 (1972) 243.
4 W. Sadee, M. Dagcioglu and S. Riegelman, J. Pharm. Sci., 61 (1972) 1126.
5 P. Neubert and K. Koch, J. Pharm. Sci., 66 (1977) 1131.
6 M.J. Tidd, L.E. Ramsay, J.R. Shelton and R.F. Palmer, Int. J. Clin. Pharmacol., 15 (1977) 205.
7 J.W. Funder, P. Feldman, E. Highland and I.S. Edelman, Biochem. Pharmacol., 23 (1974) 1493.

Journal of Chromatography, 163 (1979) 236—238
Biomedical Applications

CHROMBIO. 325

Note

Determination of antipyrine in plasma by reversed-phase high-performance liquid chromatography

THOMAS M. CAMPBELL, ELIZABETH W. MURDAUGH and PAUL G. KILLENBERG*

Liver Service, Division of Gastroenterology, Department of Medicine, Duke University Medical Center, Durham, N.C. 27710 (U.S.A.)

(Received November 16th, 1978)

The kinetics of the elimination of antipyrine (phenazone) from plasma has been used as an index of hepatic microsomal drug metabolism [1—4]. Interest in the detection of antipyrine in biologic fluids has lead to the description of several new methods for the quantitative measurement of this drug [5—7]. Recently Eichelbaum and Spannbrucker [8] have described a rapid and sensitive method for the measurement of antipyrine by high-performance liquid chromatography (HPLC). Their method requires extraction of 0.5-ml samples of plasma or saliva with dichloromethane at alkaline pH prior to chromatography over silica gel.

We report an alternative method for the measurement of antipyrine using reversed-phase HPLC. This method uses smaller aliquots of plasma (0.05—0.20 ml) and does not require extraction into organic solvents. Chromatography is performed at acid pH over bonded octadecylsilane with benzoic acid as an internal standard. The precision, accuracy, and sensitivity of this method is comparable to that reported by Eichelbaum and Spannbrucker.

EXPERIMENTAL

Chemicals

Antipyrine, N.F. was purchased from Mallinckrodt (St. Louis, Mo., U.S.A.). Benzoic acid and acetonitrile were purchased from J.T. Baker (Phillipsburg, N.J., U.S.A.) as "Baker Analyzed Grade". Methanol was "Spectroanalyzed Grade" from Fisher Scientific (Fairlawn, N.J., U.S.A.).

*To whom correspondence should be addressed.

Apparatus

The chromatographic system consists of a Milton Roy pump (Glass Engineering, Houston, Texas, U.S.A.); a Model U6K Universal Injector and Model 440 Ultraviolet Detector (Waters Assoc., Milford, Mass., U.S.A.). Ultraviolet absorption is measured at 254 nm. Samples are chromatographed at ambient temperature (21—24°) over a 300 × 4 mm I.D. stainless-steel column packed with μBondapak C_{18} (10 μm) (Waters Assoc.). The mobile phase is acetonitrile—acetic acid (1% in water) (35:65) at a flow-rate of 1 ml/min (pressure, 68 bar).

Procedure

1.6 ml of methanol was added to a glass tube containing 0.05—0.20 ml of plasma (0.5 U heparin sodium, USP, per ml) and 10 ng of benzoic acid (in 50 μl of water). The volume was adjusted to 2.0 ml with water. After mixing on a vortex mixer for 15 sec protein was removed by centrifugation at 3,000 *g* for 10 min. The supernatant was filtered through a 0.6-μm paper filter (Millipore, Bedford, Mass., U.S.A.), and 25 μl of the filtrate were injected into the chromatographic system.

RESULTS AND DISCUSSION

With the above procedure, the retention time of antipyrine was 5.5 min and of benzoic acid 7.7 min (Fig. 1). Column efficiency (HETP) for antipyrine was 0.4 mm and for benzoic acid, 0.1 mm. (Antipyrine was as easily measured at lower ratios of acetonitrile to acetic acid but the retention times were increased.)

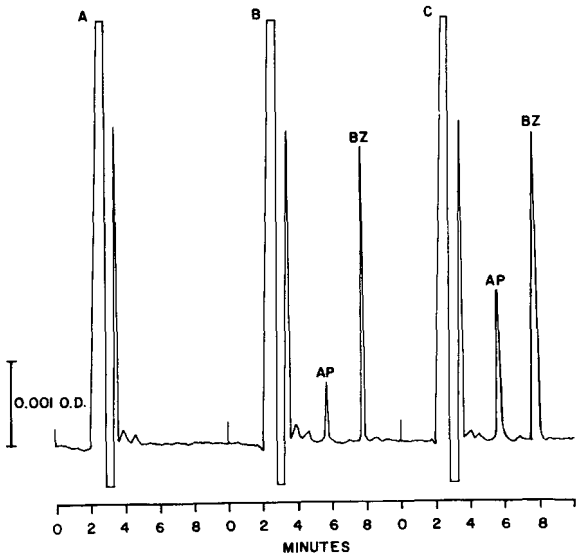


Fig. 1. Chromatograms of plasma containing antipyrine (AP) and benzoic acid (BZ). 50 μl of plasma containing (A) 0, (B) 8 and (C) 20 μg/ml antipyrine (AP) were treated as noted in the text. Benzoic acid was added to (B) and (C) as internal standard. 25 μl of the diluted and deproteinized plasma were injected. Absorbance at full scale deflection was 0.005 O.D.

Optical density was linear with concentration between 0.5 and 80 μg antipyrine per ml of plasma. Using the peak-height ratio method of quantification, the coefficient of variation for 10 replicate samples of plasma was 2.3% at 1 μg/ml and 2.1% at 8 μg/ml with 0.05-ml plasma samples; and 0.1% at 8 μg/ml and 0.1% at 20 μg/ml with 0.20-ml samples. Precision over a 45-day period was ± 5.5% (n = 16) using 0.05-ml aliquots of plasma (19 μg/ml) obtained from a subject 4 h after receiving 18 mg antipyrine per kg body weight by mouth.

Recovery of antipyrine added to plasma was linear in the range 1—8 μg/ml (0.05-ml samples) and 8—80 μg/ml (0.20-ml samples). The correlation coefficient for each group was greater than 0.99. The slope of the regression line was 1.02 ± 0.01 for the 0.20-ml samples and 1.08 ± 0.06 for the 0.05-ml samples.

We have performed over 200 determinations of antipyrine in human plasma with this method. No interfering peaks were noted in plasma from patients receiving a variety of drugs including salicylates, hydrochlorothiazide, prednisone, or insulin. 4-Aminoantipyrine is detected by this method with a retention time of 5.9 min. However, its molar extinction coefficient at this pH is less than 3% of that of antipyrine. The more polar antipyrine metabolites do not interfere with the antipyrine or benzoic acid peaks. In two separate patient studies, we compared the rate of elimination of antipyrine from plasma derived from assays using 0.05-ml and 0.20-ml aliquots. The apparent first-order rate constants for the elimination of antipyrine from plasma were 0.0422 and 0.0491 h^{-1} with 0.20-ml specimens and 0.0452 and 0.0475 h^{-1} with 0.05-ml specimens, respectively.

We believe that this method for quantification of antipyrine offers a useful alternative to that of Eichelbaum and Spannbrucker [8]. Our method requires smaller sample volumes and may be more suitable for studies in laboratory animals or pediatric patients. This method also eliminates the requirement for quantitative extraction into organic solvents and may prove useful for simultaneous determination of antipyrine and other compounds.

ACKNOWLEDGEMENTS

The authors wish to thank Robert D. Stevens, Joan T. Jordan and David Kreger for their help during this study. This work was supported in part by the National Cooperative Gallstone Study, N.I.H. contract No. 1-AM-3-2216.

REFERENCES

1 E.S. Vesell and J.G. Page, J. Clin. Invest., 48 (1969) 2202.
2 M. Lichter, M. Black and I.M. Arias, J. Pharmacol. Exp. Ther., 187 (1973) 612.
3 J. Elfstrom and S. Lindgren, Eur. J. Clin. Pharmacol., 7 (1974) 467.
4 M. Eichelbaum, G. Bodem, R. Gugler, Ch. Schneider-Deters and H.J. Dengler, New Engl. J. Med., 290 (1974) 1040.
5 R.M. Welch, R.L. deAnglis, M. Wingfield and T.W. Farmer, Clin. Pharmacol. Ther., 18 (1975) 249.
6 R.L. Chang, A.W. Wood, W.R. Dixon, A.N. Conney, K.E. Anderson and A.P. Alvares, Clin. Pharmacol. Ther., 20 (1976) 219.
7 P.J. Meffin, R.L. Williams, T.F. Blaschke and M. Rowland, J. Pharmacol. Sci., 66 (1977) 135.
8 M. Eichelbaum and N. Spannbrucker, J. Chromatogr., 140 (1977) 288.

Journal of Chromatography, 163 (1979) 239—243
Biomedical Applications

CHROMBIO. 327

Note

Rapid, sensitive determination of dipyridamole in human plasma by high-performance liquid chromatography

JOCHEN SCHMID, KLAUS BESCHKE, WILLY ROTH, GERHARD BOZLER and FRIEDRICH WILHELM KOSS

Dr. Karl Thomae GmbH, Department of Biochemistry, Birkendorfer Str., D-7950 Biberach 1 (G.F.R.)

(Received December 5th, 1978)

Dipyridamole is widely used as coronary vasodilator. Further, in the last years the combination of acetylsalicylic acid with dipyridamole is increasingly being used as an antithromboticum. The study of the bioavailability of dipyridamole in various pharmaceutical formulations as well as in combination-drugs, as mentioned above, requires specific and sensitive plasma level evaluations.

Quantitative analysis of dipyridamole in biological fluids has been performed by fluorescence measurements [1, 2]. These direct determinations without chromatographic separation do not distinguish between other fluorescent substances (metabolites and other drugs). A newer technique [3] allows the separation of glucuronides. An especial advantage of a chromatographic technique is that it permits the use of an internal standard. This improves the precision of the determination and facilitates the handling of small sample volumes. A recent publication [4] describes the detection of dipyridamole by means of high-performance liquid chromatography. The sample preparation described there is more complicated and the UV detection is less specific and sensitive.

The aim of this study was to develop a rapid, simple and specific method for determining dipyridamole in small plasma samples.

EXPERIMENTAL

Reagents and chemicals

Dipyrimidamole (R-A 8 BS) and the internal standard methoxy-dipyridamole (R-A 764 BS) were of analytical grade (Dr. Karl Thomae, Biberach, G.F.R.) (see Fig. 1). Dichloromethane, sodium hydroxide, methanol and tris(hydroxymethyl)aminomethane (Tris) were of p.a. quality (E. Merck, Darm-

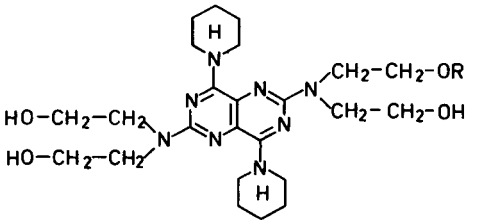


Fig. 1. Structural formulae of dipyridamole (R-A 8 BS), R = H, and the internal standard methoxy-dipyridamole (R-A 764 BS), R = OCH_3.

stadt, G.F.R.). The Tris—HCl buffer was 0.2 *N* (in water), pH 8.6. ^{14}C-labelled dipyridamole (specific radioactivity 3.75 mCi/mmole ($2.75 \cdot 10^5$ $sec^{-1} \cdot mg^{-1}$) was synthesized in the isotope laboratory of the Biochemical Department of Dr. Karl Thomae.

Apparatus

Chromatograph: Perkin Elmer 601. Detector: Spectrofluorimeter 801 Farrand Optical, excitation 415 nm, emission 478 nm. Integrator: Hewlett-Packard 3370B. Recorder: Perkin Elmer/Hitachi 56. Column: 125 × 4.6 mm I.D., filled with LiChrosorb RP-18, 5 μm (E. Merck). Pressure 1100 p.s.i. temperature 20°. Mobile phase: methanol—0.2 *M* Tris · HCl buffer (80:20), at a flow-rate of 1 ml/min.

Collection of samples

Blood samples were withdrawn with heparinised Sarstedt Monovetten^R (Nümbrecht, G.F.R.) and centrifuged to obtain plasma.

Analytical procedure

A 0.2-ml aliquot of plasma (range 0.1—0.5 ml) was pipetted into a 15-ml glass-stoppered test-tube, and 1 ml of 1 *N* sodium hydroxide and 10 ml dichloromethane, containing 100 ng internal standard, were added. After mixing for 10 min on a shaking machine the upper phase was withdrawn and discarded. The organic phase was filtered through a piece of filter-paper into another test-tube and evaporated to dryness under a gentle nitrogen stream in a water-bath at 30°. The residue was reconstituted with 50 μl of the solvent mixture and injected into the chromatograph. The amount of plasma taken for analysis should be adjusted according to the dosage. For dosings of 50 mg dipyridamole, 200 μl plasma are appropriate.

Calibration curve

Standards corresponding to 0, 10, 20, 40, 80, 100, 200, 400 ng of dipyridamole dissolved in 20 μl methanol were added to 0.5 ml plasma. After incubation for 1 h at 37° the analysis was performed as described above. The volume of 0.5 ml plasma was taken to show the absence of interfering peaks even for large plasma volumes.

Experiments demonstrating high specificity

To make sure that the dipyridamole peak was not overlapped by a metabolite with the same chromatographic behaviour, the eluate from the column

was re-chromatographed on silica gel G thin-layer plates (No. 5271, E. Merck) in the solvent systems toluene—isopropyl alcohol—ethanol—ammonia (70:15:15:1) and *n*-butanol—methyl ethyl ketone (80:20). The R_F values of dipyridamole are 0.60 and 0.80, respectively. The plates were dried in a stream of cool air and then inspected under UV light (254 nm).

Recovery

The recovery was established by liquid scintillation counting of ^{14}C-labelled dipyridamole. The labelled compound was added to plasma as described above. Following phase-separation the amount of radioactivity of the two phases was measured by liquid scintillation counting (Packard Tri-Carb, Model 3380).

RESULTS AND DISCUSSION

Sensitivity

The fluorescence detector enabled us to detect less than 200 pg of dipyridamole, as shown in Fig. 2. The detection limit in plasma is lower than 5 ng/ml as shown in Fig. 3.

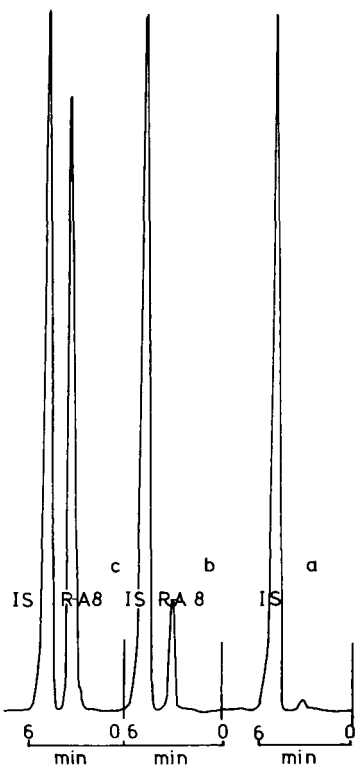


Fig. 2. Chromatograms showing (a) 500 pg and (b) 100 pg of pure dipyridamole.

Fig. 3. Chromatograms of dipyridamole showing human plasma blank with (a) internal standard; (b) 20 ng/ml R-A 8; (c) 160 ng/ml R-A 8.

Selectivity

The thin-layer re-chromatography of the high-performance liquid chromatography (HPLC) eluate demonstrated the absence of metabolites with the same chromatographic behaviour on the reversed-phase column. Ten different human plasma samples obtained from heparinized, citrate-treated and EDTA-treated blood showed practically no interference in the chromatogram.

Calibration curve

The calibration curve is linear between 10 and 400 ng dipyridamole. It is reasonable to adjust the plasma volume corresponding to this concentration range.

Precision and accuracy

Within-day precision was established on a drug-free plasma. Dipyridamole was added at a concentration of 100 ng/ml, 200 ng/ml and 400 ng/ml (Table I).

Day-to-day precision was established by analysing drug-containing plasma samples of a pharmacokinetic experiment and analysing them on different days (Table I).

TABLE I

PRECISION OF DIPYRIDAMOLE DETERMINATION ON ONE DAY (A) AND BETWEEN DAYS (B)

C.V. = coefficient of variation.

	Plasma level (ng/ml)	Mean peak ratio	C.V. (%)	No. of determinations
A	100	0.426	3.5	4
	200	0.861	0.8	4
	400	1.707	1.7	4
B	580	2.466	1.3	6

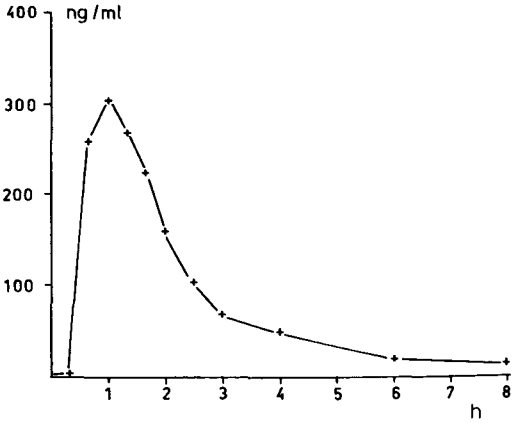


Fig. 4. Plasma concentration of dipyridamole in a human subject following peroral administration of 25 mg.

Using liquid scintillation counting, the recovery of ^{14}C-labelled dipyridamole was 88 ± 3.8% (n = 5) at a level of 100 ng/ml. For a single-step extraction, this result is excellent.

Application

Fig. 4 shows the plasma concentration of dipyridamole in a human subject following peroral administration of 25 mg dipyridamole as a tablet. This plasma concentration level agrees with the total fluorescence measurement.

This method is very suitable for routine analysis of bioavailability studies, as it is simple (due to the selective extraction), rapid (because of the short retention times), precise (on using an internal standard), specific (only dipyridamole is detected) and sensitive (with the fluorescence detector). One person is able to perform 40 analyses a day. The results demonstrate that HPLC with fluorescence detection has a high sensitivity and high selectivity, they further give evidence that HPLC is an important tool for pharmacokinetic studies.

REFERENCES

1 G. Beisenherz, F.W. Koss, A. Schüle, L. Gebauer, R. Bärisch and R. Fröde, Arzneim.-Forsch., 10 (1960) 307.
2 S.B. Zack, H.H. Tallan and G.P. Quinn, J. Pharmacol. Exp. Ther., 141 (1963) 393.
3 G. Bozler, personal communication.
4 A.K. Pedersen, J. Chromatogr., 162 (1979) 98.

Journal of Chromatography, 163 (1979) 244—246
Biomedical Applications

CHROMBIO. 328

Note

Determination of practolol in plasma by high-performance liquid chromatography

M.J. COOPER and B.L. MIRKIN*

Department of Pharmacology, Division of Clinical Pharmacology, University of Minnesota Health Sciences Center, 105 Millard Hall, Minneapolis, Minn. 55455 (U.S.A.)

(Received November 24th, 1978)

Practolol, 4-(2-hydroxy-3-isopropylaminopropoxy)acetanilide, is a selective antagonist of cardiac beta-receptors (β_1), and was the prototypic drug in this category. It has been used for the treatment of cardiac arrhythmias; the oral dosage ranges from 1.5 to 12 mg/kg. Few methods have been reported for the determination of practolol in biological specimens. Bodem and Chidsey [1] described a fluorometric method. Gas chromatographic methods have also been reported [2—4] in which the drug is derivatized before analysis. The present report describes a simple and rapid method for the determination of practolol in plasma, utilizing high-performance liquid chromatography (HPLC) with ultraviolet detection.

EXPERIMENTAL

Glassware was washed with concentrated nitric acid and silanized with 5% v/v dichlorodimethylsilane in pyridine.

Apparatus

The liquid chromatograph consisted of a Waters Assoc. Model 6000 pump and U6K universal injector coupled to a Schoeffel Instrument Spectroflow SF 770 multiwavelength monitor. The chromatographic conditions were as follows: column, μBondapak C_{18} (30 cm × 4 mm I.D.), particle size 10 μm (Waters Assoc., Milford, Mass., U.S.A.); mobile phase, 0.1% phosphoric acid in ethanol—water (1:9), flow-rate, 1.0 ml/min; column temperature, ambient; detection at 254 nm.

Chemicals and reagents

All solvents and reagents were analytical grade and were used without further purification.

* To whom correspondence should be addressed.

Extraction procedure

Blood samples obtained from pregnant sheep receiving 10 mg/kg of practolol were centrifuged at 4° for 10 min and the plasma was removed. Sodium hydroxide (5 *N*, 0.1 ml) and ethyl acetate (10 ml) were added to 1 ml plasma. This was agitated on a Vortex mixer for 1 min and centrifuged for 10 min. An 8-ml aliquot of the organic layer was evaporated to dryness in vacuo at 50°, and redissolved in 0.1 ml of the chromatographic mobile phase.

High-performance liquid chromatography

For samples containing high drug levels, 5-μl injection volumes were used at an attenuation of 0.1 a.u.f.s. Plasma specimens containing low concentrations of practolol were injected in volumes up to 60 μl at an attenuation of 0.02 a.u.f.s. Peak heights were used for quantitation.

RESULTS AND DISCUSSION

The chromatograms of representative sheep plasma samples are illustrated in Fig. 1. As can be seen, the control sample was free from any interfering peaks. The lower limit of detection was 50 ng/ml plasma, using 1-ml volumes and the recovery of practolol added to serum over the range of 0.05—8 μg/ml was 96.9 ± 12.7% (mean ± S.D., n = 16). The standard curves were linear in this concentration range and passed through the origin. The same procedure can also be used for human plasma and blank samples showed no interfering peaks.

This procedure has several advantages over previously reported techniques. The simple extraction and rapid elution (retention time of practolol is 6 min) enable the analysis of numerous samples a day. Moreover, the method is sensitive enough to study drug disposition in clinical situations where only small sample volumes are required. Though the previously reported gas chromatographic procedures [3, 4] can detect lower drug levels, the sample workup time is longer.

The method reported here is currently being used to study the disposition of practolol in the ovine maternal-placental-fetal model and the results will be published elsewhere.

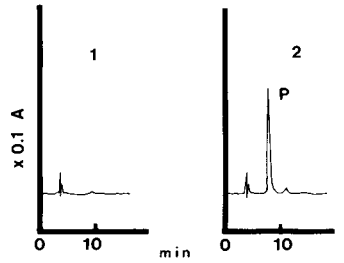


Fig. 1. HPLC of sheep plasma samples. 1, Control plasma; 2, plasma containing 7.2 μg/ml practolol (P). Conditions: column, 30 cm × 4 mm I.D. μBondapak C_{18}; eluent, 0.1% phosphoric acid in ethanol—water (1:9), flow-rate, 1.0 ml/min; column temperature, ambient; detection, 254 nm.

ACKNOWLEDGEMENTS

This work was partially supported by funds from USPHS grant HD-08580, the Minnesota Medical Foundation, the Hoffman-LaRoche Foundation and the University of Minnesota Graduate School. The technical assistance of Wilhelmina Ramos is gratefully aknowledged.

REFERENCES

1 G. Bodem and C.A. Chidsey, Clin. Chem., 18 (1972) 363.
2 B. Scales and M.B. Cosgrove, J. Pharmacol. Exp. Ther., 175 (1970) 338.
3 T. Walle, J. Pharm. Sci., 63 (1974) 1885.
4 J.P. Desager and C. Harvengt, J. Pharm. Pharmacol., 27 (1975) 52.

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc. should be on separate sheets.

Title. The title of the paper should be concise and informative. Since titles are widely used in information retrieval systems, care should be taken to include the key words. The title should be followed by the authors' full names, academic or professional affiliations, and the address of the laboratory where the work was carried out. If the present address of an author is different from that mentioned, it should be given in a footnote. Acknowledgements of financial support are *not* to be made in a footnote to the title or name of the author, but should be included in the Acknowledgements at the end of the paper.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. One original and two photocopies are required. Attention should be given to any lettering (which should be kept to a minimum) and to spacing on axes of graphs in order to ensure that numbers etc. remain legible after reduction. Axes of a graph should be clearly labelled. The figures should preferably be of such a size that the same degree of reduction can be applied to all of them. Photographs should have good contrast and intensity. Sharp, glossy photographs are required to obtain good halftones. References to the illustrations should be included in appropriate places in the text using arabic numerals. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the authors' expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. The numbers should appear in the text at the appropriate places in square brackets. In the reference list, periodicals [1], books [2], multi-author books [3] and proceedings [4] should be cited in accordance with the following examples:

1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford. New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication". The *Journal of Chromatography; Journal of Chromatography, Biomedical Applications* and *Chromatographic Reviews* should be cited as *J. Chromatogr.*

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers, Short communications and Notes will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

News. News releases of new products and developments, and information leaflets of meetings should be addressed to: The Editor of the News Section, Journal of Chromatography/Journal of Chromatography, Biomedical Applications, Elsevier Scientific Publishing Company, P.O. Box 330, 1000 AH Amsterdam, The Netherlands.

Advertisements. Advertisement rates are available from the publisher on request. The Editors of the journal accept no responsibility for the contents of the advertisements.